Viruses in the Aetiology of Rheumatoid Arthritis

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SUMMARY

The concept of an infectious aetiology of rheumatoid arthritis with special emphasis on viruses is discussed. Two main streams of current thought were considered. One was that persistent viral infection of rheumatoid synovial lining or cartilage cells could act as a target for immune destruction (target hypothesis); and the other was that viral infection of some effector cells of the immune system could lead to an autoimmune process (disordered immunoregulation).

Initially the involvement of rubella virus in the chronic inflammatory process of rheumatoid arthritis was looked at. A collection of synovial fibroblasts from patients with rheumatoid arthritis and from patients with osteoarthrosis or other non-RA disease were examined for rubella antigens by immunofluorescence and radioimmunoassay. Sections of synovial membranes and cartilages were also looked at using an immunoperoxidase technique. There was no evidence of rubella antigens in any of the cells or tissues (target hypothesis). Lymphocytes isolated from synovial fluid and peripheral blood were compared for the expression of rubella antigens and both found to be negative (disordered immunoregulation). Direct detection of rubella antigens and isolation of the virus from the cells of synovial fluid was attempted in an unusual case of rubella polyarthritis. The result was negative although the persistence of high levels of rubella IgM in this patient's serum suggested a persisting viral stimulus. The overall results provided little support for the

various hypotheses that persistent infection with rubella virus underlies the rheumatoid process.

Evidence in favour of a possible viral aetiology of rheumatoid arthritis was further sought in studying the immunoglobulins produced in vitro by cultured rheumatoid synovial membranes. These were examined for their ability to form complexes with ^{3}H-labelled viruses, namely rubella, measles, adenovirus and feline leukaemia virus. A technique was developed to detect specific in vitro ^{14}C-labelled antiviral antibodies. No antiviral activity was found in immunoglobulins associated with or produced by rheumatoid synovial membranes.

The possible involvement of retroviruses in the aetiology of rheumatoid arthritis was also investigated. Antibodies cross-reacting with primate retrovirus antigens were sought for in sera from patients with 'autoimmune' diseases by means of solid phase radioimmunoassay. There was no difference in the distribution of the immunoglobulins bound to retrovirus antigens in the three groups of patients studied: i.e. rheumatoid arthritis, systemic lupus erythematosus and a group of non-RA patients. Absorption of rheumatoid factor did not alter this conclusion. Retrovirus antigens were not expressed on rheumatoid synovial and peripheral blood lymphocytes as judged by membrane immunofluorescence, radioimmunoassay and complement-mediated cytotoxicity. The specific antiretroviral sera used in this study were produced in rabbits immunized with viral antigens grown in homologous system (rabbit cells and medium supplemented with normal rabbit

serum), avoiding non-specific immunofluorescence previously detected with donated anti-retroviral sera. Immune complexes lodged in the rheumatoid synovial membranes did not contain, and other cells within the membranes did not express, retroviral antigens. These results give a little support to the hypothesis that activation of endogenous human retroviruses or an infection with horizontally transmitted retroviruses is associated with the rheumatoid process.

The results of some of the work presented in this thesis have already been reported. The relevant references are:

(i) Hart, H. and Marmion, B.P. (1977). Rubella virus and rheumatoid arthritis. Annals of Rheumatic Diseases, 36, 3-12.

(ii) McCormick, J.N., Duthie, J.J., Gerber, H., Hart, H., Baker, S. and Marmion, B.P. (1978). Rheumatoid polyarthritis after rubella. Annals of Rheumatic Diseases, 37, 266-272.

DECLARATION

The investigations and procedures that form the basis of this thesis have been designed and carried out by the author. The complimenting experimental results when quoted are clearly marked by the researcher's name. The thesis was edited and proof-read, as English is not my native language.

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INTRODUCTION

GENERAL INTRODUCTION

Rheumatoid arthritis is a disease of our times but was probably known to our predecessors (Wood, 1976); possibly a penalty for the extension of the average life-span of mankind today. Ninety-five percent of all people are said to suffer from some form of arthritis or arthroses by the end of life, even if it is only the involvement of one joint and mostly of the degenerative variety. Of the various arthritides, rheumatoid arthritis (RA) is the most disabling and most frequently affects middle-aged females. However the disease is not confined to the older generation, young people also suffer from various forms of arthritis, mainly juvenile rheumatoid arthritis.

The prime cause(s) of adult or juvenile rheumatoid arthritis is unknown. Nevertheless during the last 10-20 years a great deal has been learnt about its clinical aspects and its intermediate pathogenesis and immunopathology; new methods have been created to facilitate diagnosis and to separate RA patients from those with other inflammatory arthritides.

Two fundamental questions remain unanswered despite these advances, viz:

a) What is the nature of the stimulus, or the cause of the loss of immunological control, that leads to the first joint or other organ changes?

and

b) What are the factors responsible for the chronicity of the

disease?

Clearly any contribution to the elucidation of these questions is very important with a disease of such high cost in terms of misery and medical treatment.

Rheumatoid arthritis is a chronic, peripheral, symmetrical polyarthritis, frequently associated with systemic manifestations including subcutaneous nodules, vasculitis, anaemia, neuropathy and pulmonary and ocular disease. The most striking feature of the disease is the chronic inflammation of the joints with eventual destruction of cartilage and bone in severe cases. Descriptions of the joint in health and in rheumatoid arthritis are numerous but attention is drawn to the review of Hamerman (1968) and to the comprehensive account of the immunopathological changes by Zvaifler (1973).

Aetiological factors have been considered in other reviews (Walton, 1968; Whaley and Dick, 1969; Ziff, 1971; Barland, 1973; Hamerman , 1975; Denman, 1975; Marmion, 1976). Research into the aetiology of rheumatoid arthritis has been a complex matter involving many disciplines of biological sciences.

At present two main - sometimes overlapping - lines of hypothesis and experimentation can be discerned. The first hypothesis postulates that the primary change in the disease is the emergence of a 'non-self' antigen(s) in the joints, or at other target sites, and that the normal immunological response to this 'non-self' antigen does not eliminate it but is chronically stimulated by it thus giving the disease. The 'non-self' antigen might be that of an external

infective agent, such as a prokaryote (bacterium, mycoplasma or chlamydia), or that of a virus. Alternatively it might be a neoantigen on a synovial lining or other stromal cell, unrelated to an infection, which arises from derepression of information in the normal complement of the somatic cell genome (e.g. an oncofoetal antigen), or by mutation in this genome.

A variant of the infective hypothesis supposes that the infective agent is present at first, then vanishes, but alters normal tissue antigens to 'non-self' during the first infective phase and that thereafter the process is self-perpetuating. Implicit in these concepts is the view that the host's immune response is normal, even if not completely effective, and that its activity is directed against an altered target cell - (presumably) outside of the effector cells of the immune system.

A second, alternative, hypothesis (Denman et al., 1976; Talal, 1975; Marmion and Mackay, 1977; Fudenberg and Wells, 1976) postulates that the primary and central change is in the effector cells of the immune system (T or B lymphocytes or macrophages) and that the rheumatoid process involves a loss of normal immunoregulatory control rather than a normal immune reaction against an altered target cell. As before such change might arise as the result of the acquisition of new genetic information by the cells of the immune system either by infection with a virus spreading laterally from person to person, or from the derepression (for reasons unknown) of a vertically transmitted, endogenous virus. Alternatively, in non infective terms, it might arise from a mutation with gain or

loss of function in an effector cell of the immune system.

Neither of these two groups of hypotheses has yet yielded decisive experimental evidence on the aetiology of RA.

In general terms, the first suffers from the defects that prokaryotic or viral infections of the joints are mostly self-limiting (i.e. the immune system is usually effective in eliminating antigens or virally altered cells); also that prokaryotes or viruses, or their gene products are not found in RA joints (see below). Further the tissue antigen altered by infection is not yet supported by the demonstration of altered cells and autoantibody.

In any case the notion of altered tissue antigens provoking a lasting autotissue response is not in line with current immunological thinking which indicates that self-reactive clones of lymphocytes (e.g. antithyroglobulin, anti-nucleic acid, anti-mitochondrial antigen, etc) are normally present in small numbers in healthy individuals, but tightly controlled. Such clones may be stimulated - for example by immunisation with tissue extracts in adjuvant or by massive tissue damage - but the autoantibody and cytotoxic lymphocyte response is of short duration (see Weir and Elson, 1969 for summary); in contrast, however, interference with thymic or T lymphocyte function may permit persistence of a heightened immune response against a 'self antigen' (Penhale et al., 1973).

The second hypothesis - deranged immunoregulation - is of more recent origin and only now coming under experimental scrutiny.

The investigations that form the subject of this thesis cover a period in which experimentation was at first based on a viral

"target cell" hypothesis and only recently have been changed to approaches resting on hypotheses of virally deranged immunoregulation.

It is necessary in reviewing the (very large) background literature relevant to the matter of the possible role of infective agents in RA, to select aspects of immunological and virological findings covering these two rather different concepts of how rheumatoid arthritis might arise.

There are numerous aspects which might be relevant to both of the major theories of the possible viral aetiology of rheumatoid arthritis. The singular involvement of the joints in the initial stages of the disease leads to a survey of the local immunopathology within the rheumatoid joint (Chapter I). Descriptions of general studies on synovial membrane and synovial fluid both '<u>in vivo</u>' and '<u>in vitro</u>' continue with discussions of autologous antigens that might contribute to the immunopathology of the rheumatoid joint.

Chapter II considers the overall systemic immunological state of the RA patients and seeks to pinpoint defects in immune function or regulation.

Chapter III deals with the existing literature on infective agents in RA, with special emphasis on viruses.

Finally, Chapter IV describes some models of viral immunopathology and considers their implications for rheumatoid arthritis.

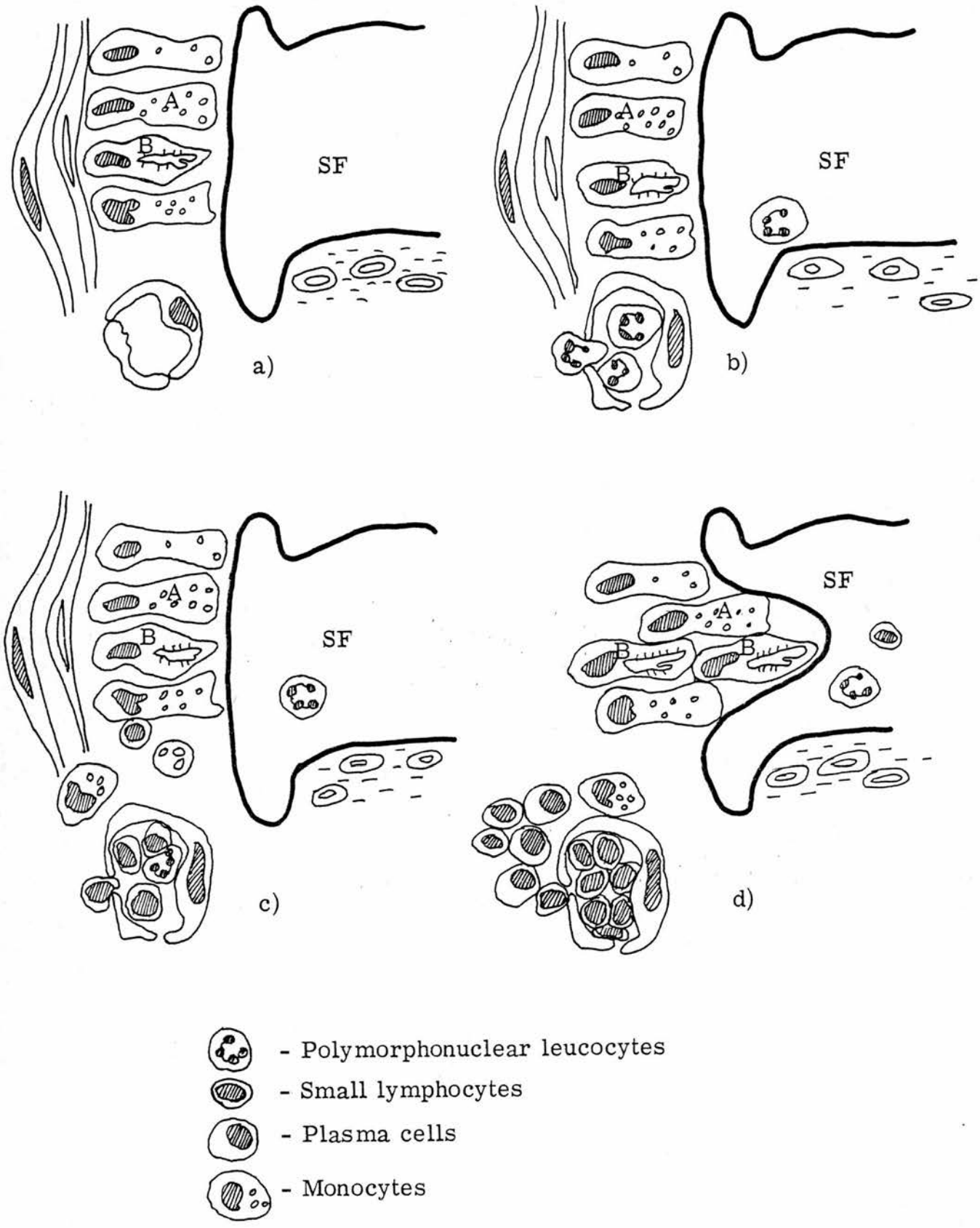


Figure 1: Schematic representation of the initial events in rheumatoid arthritis.

INTRODUCTION

CHAPTER I

IMMUNOPATHOLOGY OF THE RHEUMATOID JOINT

Histopathology and cytology of RA joint

a) Synovial membrane

Barland, Novikoff and Hamerman (1962) described electron-microscopical observations of normal human synovial membrane; these observations provide a necessary background to the initial changes of RA membrane. A supporting connective tissue formed from collagen fibres is covered with lining cells at the border of the synovial cavity. There is no basement membrane separating the lining cells from the other elements of the synovial membrane. Extracellular matrix contains amorphous material. The lining cells, clearly different from fibroblasts in the deeper layers, are divided into three groups: Type A are phagocytic with numerous filopodia and granular vacuoles filled with material similar to that in extracellular spaces; Type B have an irregular eccentric nucleus with abundant endoplasmic reticulum involved in protein synthesis; Type C are an intermediate variety of cell displaying characteristics between A and B. Other workers (Davies and Palfrey, 1971) do not agree with this division as they feel that various forms of only one cell type are represented. Capillaries with well-defined basal membranes have pinocytotic vesicles in the thicker parts of endothelial cells alternated by cells which plasma membranes appose to form fenestrae. Capillaries are seen close to the lining cells and are a normal

feature of the membrane.

Gardner (1978) summarizes the initial synovial cytology in RA. The first change identified in RA synovial membrane, as compared with normal synovial membrane (schematic Figure 1 (a)), is a dilatation of delicate capillary loops and venules with endothelial necrosis and thrombosis and slow blood flow. Polymorph leucocytes (PMNL's) marginate before adhering to venular endothelium (Figure 1 (b)). Vessels are plugged with inflammatory cells (Schumacher, 1975). They begin to move more actively through intercellular endothelial junctions and are the first cells to infiltrate the membrane. As there is no basal membrane they pass quickly into synovial fluid, but some remain scattered in the perivascular space. PMNL infiltration is transient (2-4 weeks). Increasing infiltration with blood-derived monocytes and lymphocytes then follows (Figure 1 (c)). Plasma cells were uncommon in eight patients studied within the first 6 weeks of an arthritis which later evolved into classical RA (Schumacher, 1975). The infiltrating cells remain around the vessels (Figure 1 (d)) as collections of round cells, containing lymphocytes, plasma cells and monocytes (Pearson, Paulus and Machleder, 1975). Proliferation of synovial lining cells may first be observed at this stage. From electron-microscopy (Hirohata and Kobayshi, 1964; Norton and Ziff, 1966; Kobayshi and Ziff, 1973) the impression was gained that synovial cell proliferation is probably secondary to a more fundamental immunological phenomenon that occurs in the deeper layers of the membrane and that it is associated with antigenic stimulation of the lymphoid cells. At this stage also the plasma cells appear to

increase in numbers. The lymphoid cells do not appear to pass into synovial fluids as readily as the PMNL and remain perivascularly within the membrane. The best description of late events in the lymphoid infiltration in patients with fully developed, definite, and classic RA is given by Norton and Ziff (1966). They found plasma cells and lymphocytes to be the most frequent cells in the densely infiltrated areas of the subsynovium, chiefly in the perivascular location. In some areas, the lymphocytes are the most numerous cell type, whereas in others, plasma cells predominate. They also describe transitional areas, in which both lymphocytes and plasma cell are present, together with macrophages in a higher concentration than elsewhere in the subsynovium. This important cellular interrelationship which (presumably) relates to the blast transformation of lymphocytes is well summarized by Pearson, Paulus and Machleder (1975). In parallel with the lymphoid infiltration there is an increase in the number of dilated capillaries. There is also an increase in dense bodies, endoplasmic reticulum and numbers of mitochondria in the lining cells; these events are thought to be secondary.

As the lesion progresses there is no remission of the inflammatory process. The pannus formation, i.e. the replacement of both articular cartilage and osteoporotic bones by granulation tissue, containing many lymphocytes, PMNLs, plasma cells and monocytes, begins and continues to encroach into and over the hyaline articular cartilage. Electronmicroscopic examination of the cartilage-pannus junction reveals three different pathological

features: 1) proliferation of blood vessels surrounded by a cellular infiltrate penetrating deeply into cartilage with degeneration of cartilage around invading cells 2) direct invasion of cartilage by phagocytic and fibroblastic cells with polymorphs usually absent 3) fibrous pannus directly covering cartilage surface (Kobayshi and Ziff, 1975). There was no evidence that chondrocytes were damaged or that they had initiated cartilage destruction around themselves.

It is thought that all the stages of the destructive process of RA can be linked to the initial, striking infiltration with lymphoid cells and to inflammatory factors released by them, and need not be discussed further in relation to the subject of this thesis.

b) Synovial fluid

Changes in the synovial fluid reflect the order of events in the membrane. The volumes of the synovial fluid are much increased by comparison with the minute amount in the healthy joint. The numerous PMNL's appear first and remain the principal cell in the well developed RA case. With time monocytes and lymphocytes become common, eosinophils are rare. Chemical analysis reveals the presence of various proteins, cell debris (for summary see Gardner, 1978), but as this is not of direct relevance to my purpose only the cell content of RA synovial fluid will be considered. Collins (1949) showed the range of cell population from analysis of 77 synovial fluids: polymorphs (26-95% with av. 78%), lymphocytes (3-68% with

av. 17%), monocytes (0.3-14%) and synovial lining cells (0-18%).

Lymphoid cells and immune products in the synovial membrane

The rheumatoid synovial membrane is the site of intense immunological activity. This activity is reflected in synovial fluid and eventually in the peripheral blood of RA patients. Much of the immunoglobulin synthesis and lymphoid cell activity within the membrane can be linked to the presence, or formation of rheumatoid factors of various immunoglobulin classes.

The involvement of synovial membrane in immunoglobulin synthesis has been shown by immunofluorescent studies on deposits of IgG and complement components that are to be found mainly in the cytoplasm of the phagocytic lining cells (Kinsella, Baum and Ziff, 1969; Rodman et al., 1967). The localization of immunoglobulin and complement in the same sites suggests that the immunoglobulin has been fixed to an antigen. Patients with circulating rheumatoid factor showed staining for additional immunoglobulins (IgM/IgA).

The rheumatoid synovial membrane can synthesize IgG, IgM and IgA in vitro in quantities approaching those of normal spleen (Smiley, Sachs and Ziff, 1968). It has been calculated that, in vivo, the intact RA synovial membrane synthesizes an amount of Ig equal to 12 to 26% of the total in the serum (Sliwinski and Zvaifler, 1970). In some illuminating experiments Herman et al. (1971) immunised patients with tetanus toxoid and then exposed explant cultures of their synovial membrane (previously removed by synovectomy) to the antigen. Specific antibody to tetanus toxoid was not formed and this apparently limited response of the synovium to an

exogenous antigen, despite its large immunoglobulin production, suggested to the authors a prior commitment of the synovial lymphoid infiltrate to an antigen already present in the synovial membrane. From animal models of chronic synovitis induced by the intra-articular injection of antigen (in previously immunized rabbits) there is evidence to support this notion. 30 to 40% of the immunoglobulins produced by the inflamed synovial membrane is a specific antibody to the inducing antigen (Cooke and Jasin, 1972).

A study of eluates from the RA synovial membrane indicates that the immunoglobulins synthesized have light chain restriction as illustrated by different κ and λ composition of the IgG chains expected from the levels in sera (Lindström, 1970). Less frequently oligoclonal patterns of IgG in rheumatoid synovial fluid, absent from autologous serum, are found which suggests restriction in the heterogeneity of immunoglobulins produced by synovial membrane (Mellbye, Naes and Munthe, 1976). In one case of atypical RA measles-virus specific antibodies were absorbed from the oligoclonal synovial fluid IgG (Vandvik, Mellbye and Norrby, 1977).

The operation of cell mediated immune mechanisms, as well as local immunoglobulin synthesis, is suggested by electronmicroscopy observations of large numbers of lymphoblasts in association with macrophages in the subsynovial lymphocytic infiltrates (Kobayshi and Ziff, 1973; Neumark and Farkas, 1973; Ziff, 1974); a possible morphological marker of cellular hypersensitivity. Lymphocytes with cytotoxic activity for cell culture monolayers or allogeneic

synovial fibroblasts isolated from synovial membranes and fluids have been identified (Hedberg and Kallen, 1964; MacLennan and Loewi, 1968; Panayi, 1976).

The antigen responsible for the activation of the cell-mediated immunity was present in rheumatoid synovium (Bacon et al., 1973). The majority of rheumatoid patients' peripheral blood lymphocytes responded with blast formation to extracts of both autologous and homologous rheumatoid synovial membranes, but not to antigens in psoriatic or osteoarthritic synovial membranes. Pity, that crude extracts of synovial membranes were used and there was no attempt to further identify the nature of the antigen responsible for the cell-mediated immunity.

Immunological investigations of synovial fluid

Evidence of interaction of immunoglobulins and sensitised cells with antigen is also found in RA synovial fluids. A characteristic feature of joint fluids is the presence of intermediate 9S-17S IgG complexes (Winchester, Agnello and Kunkel, 1970).

The haemolytic complement activity in the synovial fluid of RA patients is lower than would be expected from the serum levels (Hedberg, 1964; Pekin and Zvaifler, 1964) where the haemolytic activity is normal or slightly elevated (Vaughan, Bayles and Favor, 1951; Ellis and Felix-Davies, 1959). Reduction of the intra-articular concentrations of C_1 and C_4 (Zvaifler and Pekin, 1963) and C_2 (Fostiropoulos, Austen and Bloch, 1965) was shown to be proportional to the total decrease of the haemolytic complement.

These findings are consistent with immune activation of the complement sequence, by IgG complexes in the fluid (Winchester, Agnello and Kunkel, 1970). A predominance of immunoglobulin with λ-type chains, similar to that in membranes, has been reported in RA synovial fluids (Epstein and Tan, 1966).

The synovial fluid also reflects the cell-mediated immune reactivity. The expression of effector functions of cell-mediated immunity appears to be mediated by lymphokines produced by sensitized lymphocytes (Dumonde et al., 1969). Lymphokine-like materials, which include macrophage migration inhibitory factor, lymphocyte blastogenic factor (Andreis, Stastny et al., 1974) and immunoglobulin synthesis-enhancing factors (Rosenthal, Stastny and Ziff, 1974) have all been found in synovial fluids and supernatants of explants of inflamed synovial tissue.

Role of rheumatoid factors in the immune response of synovial membranes

IgM rheumatoid factor is the serological abnormality most consistently found in association with RA and has been described in numerous reviews (Kunkel and Tan, 1964; McCormick, 1975); it is beyond the scope of this introductory survey to go into details. In spite of an initial excitement that RF would provide a clue to the nature of the initial immunopathological process, the exact sequence of events which stimulates the production of rheumatoid factors is still unknown.

IgM rheumatoid factor has an affinity for autologous and homologous immunoglobulins, mainly IgG, but this reaction is weak compared to the reaction for aggregated IgG and even for heterologous immunoglobulins. For a long time it was believed that the higher

reactivity was due to increased affinity for aggregated IgG supposedly exposing new antigenic determinants, but recent demonstration of comparable avidities of Fab fragments of IgM RF with monomeric and polymeric IgG shows that increased binding of 19S IgM RF is due to the multivalency of the IgM molecule (Dissanayake, Hay and Roitt, 1977). The question of just what form the autologous gammaglobulin is in when it acts as an antigen in the production of IgM RFs has intrigued a number of workers. From the humoral studies, the IgM rheumatoid factors are now believed to result from the autoimmunization to IgG bound in immune complexes (Williams and Kunkel, 1963; Mellbye and Natvig, 1970). This view is also supported by studies on cellular immunity. Migration inhibition, a close 'in vitro' correlate of delayed hypersensitivity was shown with heat-aggregated or antigen-complexed IgG, including measles virus and antibody complex (Eibl and Sitko, 1975). The apparently specific cell-mediated immunity to mycoplasma antigens was also shown to be due to the serum immunoglobulins absorbed from the culture medium (Maini et al., 1975; Brostoff and Roitt, 1975). Autologous IgG did not stimulate lymphocytes to produce migration-inhibition factors (Fröland and Gaarder, 1971). The formation of IgG complexes has been assumed to alter or expose antigenic sites of IgG molecules and this in turn stimulates the production of IgM rheumatoid factor. More experimental evidence is needed for this assumption, but at present the IgG immune complexes indeed appear 'in vivo' to be the substrate for IgM rheumatoid factor. The nature of IgG immune complexes has become clearer from the illuminating work of Pope and his colleagues

(Pope, Teller and Mannik, 1975; Mannik, Pope and Teller, 1975). They have shown that the intermediate IgG immune complexes are in a form of self-associated IgG rheumatoid factors that form cyclic dimers (Pope, Teller and Mannik, 1974). This leaves two antibody sites free for further aggregation of the dimers or for further reactions with monomeric IgG and aggregated IgG. It seems that the existence of IgG rheumatoid factors has been masked by (1) their high affinity of association with each other rather than with monomeric IgG (Pope et al., 1974) and (2) by the fact that most routine diagnostic techniques detect only IgM rheumatoid factors. Lymphocytes separated from disaggregated synovial membranes were shown to produce IgM rheumatoid factor 'in vitro' by means of the haemolytic plaque assay system employing sheep erythrocytes (SRBC) sensitized with reduced and alkylated rabbit IgG anti-SRBC antibody as target cells (Taylor-Upsahl, Abrahamsen and Natvig, 1977). These were observed in all of the synovial tissue cell preparations from seropositive patients, but not from seronegative and thus the test was specific for IgM RF. It would be interesting to know whether the self-associating IgG rheumatoid factors are also produced locally as elucidation of the events leading to their formation is central for an understanding of the early events in the synovial inflammation of RA.

Local synthesis of IgG-anti Ig seems likely from Smiley et al.'s (1968) work as they showed that main class of immunoglobulin synthesized by rheumatoid membrane was IgG (79% of total). This

fits with the results of Kinsella, Baum and Ziff, (1969)and Rodman et al. (1967) who found by immunofluorescence that in the synovial membrane of RF seronegative patients, the cytoplasm of phagocytic cells and those in perivascular sites (McCormick and Day, 1963), stained for IgG; possible local synthesis of the IgG rheumatoid factors. Natvig and his colleagues cleverly realizing that removal of the Fc regions by pepsin would liberate the hidden binding sites, observed that as many as 25-60% of the plasma cells in the synovium displayed an anti-IgG specificity following treatment with this enzyme (Natvig, Munthe and Pahle, 1975; Natvig and Munthe, 1975). This antibody appears to be the dominant antibody activity exhibited by plasma cells in both seronegative and seropositive cases of active rheumatoid arthritis. RFs also show the light chain restriction. The restriction extends from a gross kappa preponderence to the finer restriction involving the kappa III b sub-subgroup (Kunkel et al., 1974). Interestingly all classes of RFs also reveal acidic pI (isoelectric point) values that substantially differ from other Ig molecules (Trieshmann, Abraham and Santucci, 1975).

It thus seems that the bulk of the immune reactivity in the synovial membrane, both in humoral and cell-mediated effector arms, might be directed to the formation of the various rheumatoid factors, but other specificities have not been excluded. The specificity of the immunoglobulins produced by those synovial plasma cells not engaged in RF production remains an interesting problem, wherein may lie one important clue to the aetiology of RA.

The formal possibilities are that they might be antibodies against exogenous antigens (infective agents) or against endogenous (autologous) antigens other than the Fc portions of IgG. In crude synovial membrane extracts a variety of antigens have been identified. These include collagen, cartilage, nuclear antigens, and CEA-like substance. They all could conceivably contribute to the intense immunological activity of the rheumatoid synovial membrane and are therefore reviewed in this chapter. The former possibility of antibodies against exogenous antigens is referred to in Chapter III.

Autologous antigen-antibody reactions in RA

a) autologous nucleic acids and nucleoproteins

Antinuclear antibodies (ANF) are common in the serum of patients with RA and they may be present at an even greater concentration in joint fluids (Barnett, Bienenstock and Bloch, 1966; Elling, Graudal and Faber, 1968) thus suggesting either local production, or concentration in the extravascular space. The prevalence of ANF in RA has been variously reported as low as 10% (Friou, 1958) or as high as 65% (Alexander, Bremner and Duthie, 1960). It is now clear that the choice of nuclear substrate used for detection of antinuclear factors is very important, as some ANF have a restricted range of activity. 64% of RA sera and 62% of paired joint fluids reacted predominantly or solely with human polymorphonuclear nuclei (Elling, Graudal and Faber, 1968). The granulocyte-specificity (GS) of antinuclear factors has been confirmed by others (Wiik, Jensen and Friis, 1974) who found the

predominant ANF to be IgG GS-ANF (74.1% of RA sera).

ANF has been eluted from synovial membranes in a small proportion of RA patients by using a high molarity salt elution method (Munthe and Natvig, 1971). When a longer elution procedure was adopted (10 hours) the antinuclear factors were uncovered in a larger proportion of RA eluates (37%) (Cracchiolo and Goldberg, 1972).

Antinuclear activity has been also found in the macroglobulin fraction (19S) thus differing from SLE ANF which is associated predominantly with 7S gammaglobulins (Baum and Ziff, 1962). The association of rheumatoid factor with antinuclear activity was demonstrated in all seropositive RA patients tested (McCormick and Day, 1963; Hannestad and Johannessen, 1976).

The specificity of the antinuclear factors found in RA is even more complicated to assess. The following nuclear antigens have been clearly identified as reacting with separate antinuclear antibodies: (1) Deoxyribonucleic acid (DNA), (2) histones, (3) nucleoprotein and (4) phosphate extracts (usually nuclear membrane antigens).

Antibodies to double-stranded, undenatured, DNA (2DNA or nDNA), prepared from the KB cell line or calf thymus nuclei, are very rarely detected in RA patients (Johnson, Edmonds and Holborow, 1973; Rochmis et al., 1974; Parker, 1973); this characteristic absence of anti-nDNA antibodies helps to distinguish RA and other conditions from SLE.

If the antinuclear factors found frequently in RA patients are not directed against nDNA then the other possibility is that the nucleoproteins act as an antigen substrate.

Soluble nucleoproteins are indeed regularly found in the synovial exudates and antibodies to them can be detected by radioimmunoassay (Robitaille and Tan, 1972); also antibodies to denatured DNA (single-stranded) were found in sera of 50% RA patients by radioimmunoassay. These antibodies were not specific for RA and were found commonly in other inflammatory arthritides, perhaps as a result of tissue destruction.

Oval, basophilic, Feulgen-positive globular particles are specific for RA. These DNA globules appear to represent nuclear debris from degenerating cells and are found free in the synovial fluid or adherent to the membranes of polymorphs (Pekin, Malinin and Zvaifler, 1966; Malinin, Pekin and Zvaifler, 1967).

RA synovial fluids may also form cryoprecipitates - immune complexes readily precipitable with prolonged standing of synovial fluids at 4°C. These cryoprecipitates contain DNA as detected by the diphenylamine reaction (Marcus and Townes, 1971; Cracchiolo et al., 1971) and self-associating IgG rheumatoid factors. The intriguing observation has been made that Fab regions of individual rheumatoid IgG molecules can have both anti-human IgG and anti-nuclear activity; in some seropositive RA sera all the antinuclear activity could be removed with absorption of RF (Hannestad and Johannessen, 1976). The favoured interpretation of how this might arise was that the structural

similarity between IgG and the nuclear antigen stimulated the formation of polyclonal (rarely oligoclonal) antibody.

b) cartilage antigens

A major consequence of rheumatoid joint inflammation is the degradation and destruction of articular cartilage. Although it has been recognized that cartilage has two distinct antigenic determinants there have been few attempts to link these in the causation or perpetuation of RA.

Herman, Wiltse and Dennis (1973) tested rheumatoid synovial fluid cells with a rabbit anti-PPC- and anti-PPL-sera (PP-protein-polysaccharide, C-costal cartilage material, L-light fraction cartilage material) and found the rabbit sera reacted with the cellular antigens. However, they failed to demonstrate antibody to these proteinpolysaccharides in patients' sera in the antibody eluted from rheumatoid synovial membranes and in immunoglobulin synthesized *in vitro* by this tissue. They did demonstrate a mitogenic response of peripheral blood lymphocytes to allogeneic cartilagenous antigens in 34% of RA patients. Destruction of cartilage was noted by X-ray examination in the positive patients all of whom had the disease for at least five years. The lymphocyte response to cartilage antigens, although not specific for RA, could conceivably perpetuate joint inflammation but was not thought to initiate joint inflammation (Herman *et al.*, 1973). This conclusion fits the histopathological observations, as the invasion of cartilage is a secondary event to the initial

infiltration of the subsynovium with lymphoid cells.

c) collagen

Collagen also appears to be able to stimulate an autoimmune reaction. Antibodies to collagen in serum or synovial fluid have been found in a high proportion of RA patients and are present in both IgM and IgG classes (Andriopoulos et al., 1976). Digestion of synovial fluids with bacterial collagenase gave higher titres of anticollagen antibody and converted negative synovial fluids to positive probably as the result of the liberation of antibody complexed to collagen (Menzel et al., 1976).

There have also been isolated observations of CEA-like material extracted from RA membranes with chromatographic properties and perchloroacetic acid sensitivity different from that produced by colorectal tumours (Unger, Panayi and Lessof, 1974, 1975; Unger, Panayi and Tidman, 1977); antibody responses to this CEA have also been demonstrated in RA sera (Panayi, pers. comm.). The relationship of this observation to the collagen immune complexes should be explored.

INTRODUCTION

CHAPTER II

IMMUNOLOGICAL STATE OF RHEUMATOID ARTHRITIS PATIENTS: EVIDENCE FOR DISORDERED IMMUNOREGULATION

As background to the possible role of a persistent virus infection interacting with the effector cells of the immune system to cause disordered immunoregulation in RA one may first consider the state of the immune system of rheumatoid patients.

The most dramatic evidence that effector cells of the immune system are directly involved in pathogenesis of RA comes from observation on therapy by thoracic duct drainage. Recent studies by Paulus and his colleagues (Paulus et al., 1973; Pearson, Paulus and Machleder, 1975; Paulus et al., 1977) have shown that thoracic duct drainage produced in 7 days a subjective lessening of joint swelling, morning stiffeness, fever and in some cases the ESR became normal. Rheumatoid nodules decreased in size or disappeared. The striking decreases in IgG concentration in the first week with unchanged IgM levels were not accompanied by lowered serum RF titres. Infusion of autologous living lymphocytes into the circulation resulted in a transient exacerbation with the homing of ^{51}Cr cells to joints. Intra-articular injection of autologous cells resulted in an acute effusion and persistent inflammation of the injected joint. Thoracic duct lymphocytes contain both

T- and B- lymphocytes. There was no attempt to subdivide the lymphocyte population.

The involvement of T- and B- lymphocytes has been tested in other studies. As understanding of the clinical aspects of T- and B- lymphocytes in rheumatic diseases is of prime importance to any consideration of viral involvement in RA the lymphocytes are discussed further.

Reaction of RA lymphocytes in mixed lymphocyte reaction (MLR) and to stimulation with phytomitogens and allogeneic cells

Evidence for abnormalities of T- cell function in rheumatic diseases has been accumulating. Several investigators have described depressed delayed hypersensitivity to several antigens (Whaley et al., 1971; Phillips et al., 1976a). In SLE this is limited to periods of active disease (Rosenthal and Franklin, 1973). The blast transformation of lymphocytes of RA patients when mixed with those of unrelated donors was originally found to be diminished (Astorga and Williams, 1969); more recently the response of RA lymphocytes in mixed lymphocyte reaction (MLR) has been found to be normal (Rawson and Huang, 1974a; Caperton, Baker and King, 1975), or enhanced (Keystone et al., 1976). The differences lie in the sera used to culture the lymphocytes. Normal or enhanced transformation was observed when medium was supplemented with pooled human serum. The demonstration of suppression of MLR responses by autologous RA serum is a consistent observation. Interestingly, rheumatoid sera did not suppress the MLR of normal lymphocytes. The enhanced cellular reactivity but suppressive

serum effect may reflect an '*in vivo*' state of altered T-cell function in patients with rheumatoid arthritis.

A functional defect in cellular immunity was also observed by studying blast transformation of peripheral lymphocytes with phytohaemagglutinin (PHA), concanavalin A (con A) and pokeweed mitogen (PWM). The PHA response, thought to be specific for T-lymphocytes, was depressed, while PWM response was normal (Silverman *et al.*, 1976). The decreased mitogenic reactivity could be explained in part by significantly lower numbers of small lymphocytes contained in the preparations of RA cells (Horwitz and Garrett, 1977). Studies of the quantitative binding of phytomitogens revealed that the ability of RA lymphocytes to bind PHA was significantly lower than that of normal lymphocytes and in fact delineated two different populations of normal and RA lymphocytes (Rawson and Huang, 1974b). The measurements of responses to mitogens are however of limited value; few investigators consider the effect of serum suppressors and anti-lymphocyte antibodies in autologous serum on the mitogenic reactivity (Horwitz, Garrett and Craig, 1977).

A more interesting but little used approach would be to compare the mitogenic reactivity of synovial fluid lymphocytes with those from the peripheral blood of the same patient. Stratton (1972) found a poor synovial fluid lymphocyte response to PHA and PWM but a normal peripheral blood lymphocyte reactivity to the two mitogens, whereas Panayi (1973) found depressed reactivity with both sets of lymphocytes.

That there may be differences between the two populations of lymphocytes - i.e. peripheral blood and synovial fluid - is also indicated by the observation that synovial fluid lymphocytes (SFL) can stimulate autologous peripheral blood lymphocytes (PBL) (Crout, McDuffie and Ritts, 1976). The reaction was one way as no stimulatory effect of PBL on SFL was observed. The stimulation was specific for lymphocytes as no equivalent stimulation was produced by SF PMNs. A stimulatory substance was also present in synovial fluids as the addition of autologous cell-free synovial fluid resulted in a significantly increased incorporation of tritiated thymidine by peripheral blood lymphocytes (Okamoto et al., 1973; Kinsella, 1973; Kinsella, 1976).

It is reasonable to suppose that the stimulatory effect is due to immune complexes in RA synovial fluids (Kinsella, 1976). The one way stimulation of PBL by SFL could be explained by IgG - IgG complexes binding to SFL; RA patients have been shown to possess a larger proportion of Fc-receptor positive lymphocytes (Sharpin and Wilson, 1977a, 1977b). Bound immune complexes could also be responsible for the decreased MLR and lectin reactivity of SF lymphocytes .

However it was concluded that the inhibitory material must be different from the material on SFL that stimulates autologous PBL (Crout, McDuffie and Ritts, 1976) because treatment of PBL with RA synovial fluid decreased responsiveness to PHA, but did not differentiate the stimulation of treated and untreated PBL by SFL. The expression of altered antigens on SFL inducing

specific blast transformation of PBL has not been excluded.

Other evidence indicating that lymphocytes might be abnormal in rheumatic diseases comes from the presence of naturally occurring antibodies against lymphocytes. Mittal et al. (1970) found them in 14% patients with rheumatoid arthritis while Terasaki, Mottironi and Barnett (1970) found them in 56% of RA patients. Other workers have demonstrated lymphocytotoxins (Steffen et al., 1973; Winchester et al., 1975). Lymphocytotoxic antibodies have not been shown to have HLA specificity for the target lymphocytes (Raum et al., 1977) but appear from the work of Lies, Messner and Williams (1973) to be T-cell specific. The T-cell specific antibodies could play a significant role in controlling T-cell function and might explain the decreased reactivity of RA patients to antigens in the delayed hypersensitivity skin reaction and also the decrease in PHA binding and stimulation exhibited by RA lymphocytes.

Lymphocytotoxic antibodies (LCTAB) have been found in high frequency among consanguineous and non-consanguineous members of SLE patients' families (Malave, Papa and Layrisse, 1976; De Horatius and Messner, 1975) compared to normal controls suggesting that SLE families are more exposed than the control population to environmental causative factors, such as viral infection. A similar study has not been done in the families of RA patients; as the frequency of LCTAB is lower in RA than that in SLE patients and it might be difficult to show the trends as clearly.

T-lymphocytes might not be the only cells to contain antigens that can stimulate the production of autoantibodies. A precipitating antibody referred to as rheumatoid arthritis precipitin (RAP) was detected in 67% of RA patients (Alspaugh and Tan, 1976). The RAP reacts specifically with cell free extracts of human diploid B-lymphocyte continuous cell cultures, but not with normal untransformed peripheral blood lymphocytes. The antigen was not related to those coded by the Epstein-Barr virus and the precipitating antibody was not removed by absorption with aggregated gammaglobulin. This antigen may be related to those 'mitosis antigens' expressed in transformed cells; adsorption studies with normal human lymphocytes undergoing mitogen transformation and leukemic cells are required.

A relationship between B-lymphocyte surface antigen and RA was observed by Panayi and Wooley (1977) who tested sera from multiparous pregnant women and found three that reacted specifically with B-lymphocytes. One serum of the three reacted with B-lymphocytes from 74.4% of RA patients and with only 27% of controls. In the mouse system Ia antigens, present on B cells, are coded for within the same genetic region as genes controlling certain immune responses (Ir). Extrapolated to man this might be related to a genetic predisposition to RA. Panayi and Wooley's observations may be related to the observation of McMichael et al. (1977) of an increased frequency of HLA complex antigens CW3 and DW4 in RA.

Lymphocyte populations in RA

Numerous laboratories have estimated the percentage of B- and T-lymphocytes in the peripheral blood of RA patients and healthy individuals in the hope of unmasking an abnormality but without consistent trends. Scanty data have also been obtained on synovial fluid and membrane lymphocytes. A summary of early references is given by Messner (1974) who concludes that the percentage of B-cells in the blood is likely to be normal while that in synovial fluid appears to be lower possibly relating to disease activity. The percentage of T-cells in blood appears to be normal in the majority of cases, but may decrease in periods of increased disease activity; T-cells in synovial fluid may be normal or increased.

More recently the study of lymphocyte populations has been extended to cells dispersed from synovial membrane by enzymatic treatment (Loewi, Lance and Reynolds, 1975; Abrahamsen et al., 1975; Bankhurst, Husby and Williams, 1976) or to cells in sections of synovial membranes (Tannenbaum et al., 1975). The studies revealed that the predominant cells in the rheumatoid membrane are T-cells or null cells. The study on synovial membrane sections contributed also to the understanding of the lymphocyte organization within the membrane: B-lymphocytes are found in well developed follicles or within smaller nodules of lymphocytes. These were always surrounded by a zone of non-EAC reactive lymphocytes (T-lymphocytes or null cells).

The specificity of high T-cell and low B-cell percentages

for RA synovial fluid and membrane has been put in doubt (Putte et al. 1976). Other diseases, namely polyarthritides, show the same ratio of T- and B-cells. The phenomenon of high T-cell and low B-cell percentages seems to be related to general inflammation.

A more interesting observation related to immune reactions, rather than inflammation, is the appearance of hyperbasophilic immunoblasts (HBI) in smears of lymphocytes from RA and SLE patients. They were not found in psoriatic arthritis with acute inflammatory manifestations (Delbarre, LeGo and Kahan, 1975). These cells belong to a group of proliferating, atypical, lymphocytes. They have strongly basophilic cytoplasm and high nuclear-cytoplasmic ratio and are capable of producing immunoglobulins, thus may be considered B-lymphocytes undergoing differentiation into plasma cells. They are found frequently during the course of bacterial or viral infections, such as infectious mononucleosis. In acute viral infections the increase of HBI cells is transient (10-15 days), while in connective tissue diseases, particularly SLE, the HBI level may be high for a long period.

INTRODUCTION

CHAPTER III

SEARCH FOR VIRUSES IN RA

The search for prokaryotes as aetiological agents in RA has a long history and various claims have been made from time to time. There is, however, no clear evidence to substantiate them (Marmion, 1976) and they will not be discussed any further in the present context.

Attention will be focused on the possible role of viruses and the attempts that have been made to isolate them, or, to provide evidence of viral genetic information persisting in the RA joint.

Direct observations:

Under the electronmicroscope, viral particles, or in the case of defective viruses, accumulations of virus proteins can be seen directly in virus-infected cells.

Electronmicroscopic studies of the earliest stages of rheumatoid arthritis have received very little attention. The only effort to observe viral particles within the first month of the onset of arthritis (which later was diagnosed as classic or definite RA) was reported by Schumacher (1975). In four patients from eight studied, a variety of virus-like particles was found associated with the endothelium or perivascular cells. One of these was a

structure (100-140 nm) budding from the vascular endothelium and the other (90 nm) protruded into a vacuole. Each particle had a dense core surrounded by a lucent area and a unit membrane and thus might be considered to be retroviruses (Bernard, 1960). Schumacher stressed that non-viral cell components are very difficult to distinguish from true viruses in EM of tissue sections. This limitation of electronmicroscopy also applies to the studies of the later stages of well developed RA. Neumark and Farkas (1970) observed nuclear inclusions in rheumatoid synovial membranes, located mainly in the vascular endothelial cells and mononuclear cells; all in the deeper zones beneath the lining cell layer. The authors recognized that none of these were specific for virus infection, but they did suggest a possible virus origin. Such structures have not been seen by others (Schumacher, 1975). Bundles of 42 nm tubules have been noted in lymphocytes from one patient with rheumatoid arthritis (Hovig, Jeremic and Stavem, 1968). The paramyxovirus-like or tubuloreticular structures typically found in systemic lupus erythematosus (Gyorkey et al., 1972) are rare in rheumatoid arthritis and represent a notable difference between the two diseases. Cytochemical studies of tubuloreticular structures suggest that they contain phospholipid and acid glycoprotein and thus most probably are not viral in themselves, although conceivably they might accompany virus infection (Karpas et al., 1971; Schaff, Barry and Grimley, 1973).

Complete virions have been seen budding from the cytoplasmic membrane of RA macrophages; these cells also contained short

tubular structures (12 nm). Increased lysosomal activity was seen around the tubules. Tubulofilamentous fragments (about 16 nm in diameter) were also seen detaching from lymphoid cells (Neumark and Farkas, 1973). The small number of positive EM observations and their limitations in the differentiation of virions from cellular components calls for an additional proof of the presence of viruses. Obviously virus culture would be the most convincing.

Attempts to culture viruses

Basic virological techniques were directed initially to detect a cytopathic virus in (1) tissue culture cells co-cultivated with materials from RA joints or (2) in cultured synovial cells. The clinical material used in these studies was from patients with well developed RA.

Cultured fibroblastic synovial cells are thought to be synovial Type B or C lining cells (Smith, 1971). A more recent description of the adherent cells dissociated from synovial tissue has been given by Abrahamsen, Johnson and Natvig (1977). Initially 68-80% of non-lymphoid cells could be detected, of which 40-60% exhibited marked phagocytosis. Less than 14% of the non-lymphoid cell population could form EA rosettes and was therefore an Fc-receptor bearing cell population. 30-60% of cells were positive for collagen and the proportion increased to over 90% on prolongation of the culture, while the viability or the phagocytic ability of the cells did not alter significantly. The phagocytic ability distinguishes these cells from fibroblasts derived other

tissues, such as human skin. Other distinguishing characteristics have been reported. The RA synovial fibroblasts synthesize more hyaluronate-protein, glycoproteins of different molecular weights and more prostaglandins E (Hamerman, 1975; Robinson and McQuire, 1975). They are also said to have a longer doubling time and a shorter life expectancy than "normal" fibroblasts (Palmer, 1975).

An early systematic study for detection of cytopathic virus was carried out by Ford and Oh (1965). They cultured synovial cells for up to five months and observed no spontaneous degeneration to suggest latent viral infection. Furthermore, no cytopathic effect (CPE) was seen in primary cultures inoculated with 20 synovial fluid specimens and subjected to 5 'blind' passes at 3-week intervals. Smith and Hamerman (1969) failed to isolate virus from rheumatoid cells. Barnett (1966) inoculated Hep-2 cells, human embryonic fibroblasts and human amniotic cells with synovial fluids and subsequently searched for an 'infective agent' by indirect immunofluorescence using the patient's own serum, but results were negative. The pursuit of a cytopathic virus continued by comprehensive virological investigations of Phillips (1971) and Wilkes et al. (1973), but again no virus was isolated. This contrasts with Serre et al. (1972) who claimed to isolate a cytopathic virus after co-cultivation of synovial fluid cells with WI-38 cells. Electronmicroscopy revealed a virus of the paramyxovirus group, but no pictures were published and there has been no follow up of this observation.

Smith and Hamerman (1969) reported differing susceptibilities to Newcastle disease virus (NDV) in non-rheumatoid cells (80%

infected) as compared to RA synovial fibroblasts (30-60% infected), a difference which was statistically significant. This suggested that a noncytopathic virus with interfering activity might be present. In the same way Grayzel and Beck (1970) reported that synovial cultured cells from RA patients were resistant to rubella virus whereas similar cultures prepared from non-rheumatoid sources were susceptible. A number of attempts to repeat these important results were unsuccessful (Person, Sharp and Rawls, 1971; Runge and Allison, 1972; Parker, McCellum and Kerby, 1972; Spruance et al., 1972). Clarris, Fraser and Rodda (1974) concluded that the resistance to rubella rested on defective absorption of rubella virus, because of a higher production of hyaluronic acid coat in rheumatoid cultures rather than on interference with intracellular replication. This has since been confirmed by Patterson, Peterson, Howard and Deinhardt (1975).

This subject is particularly intriguing in view of claims of Hamerman and his associates that resistance to rubella infection can be conferred on rabbit synovial cells either by intra-articular injection of rheumatoid synovial cells into rabbit joint (Smith et al., 1974) and or in vitro by fusing rabbit and human cells with Sendai virus (Smith and Hamerman, 1974). While the explanation of the resistance to rubella might be provided by the higher hyaluronic acid production in RA cells, it is difficult to imagine the transfer of this higher hyaluronic production to rabbit cells without the mediation of an 'infective' agent. Interestingly, a higher production of hyaluronic acid has been induced in fibroblasts transformed by SV40 (Ishimoto, Temin and Strominger, 1966); also cell lines chronically infected with Rauscher leukaemia

or Rous sarcoma viruses showed a 3-5 fold increase in hyaluronic acid production (Hamerman et al., 1968).

In more direct attempts to detect a non-cytopathic virus Person, Sharp and Rawls (1973) examined cell cultures from synovial fluids and membranes after co-cultivation with Vero cells and attempted induction with IUDR. ^{3}H-uridine and ^{3}H-thymidine were added to the cultures and supernatant fluid fractionated on sucrose and caesium chloride gradients and tested for radio-labelled material at the densities normally occupied by various viruses and mycoplasma. Grayzel (1973) used a similar approach to demonstrate a non-cytopathic virus by adding ^{3}H-uridine to cultured rheumatoid cells, with or without pre-treatment with BUDR and actinomycin D. No virus was found in either investigation.

Norval and Marmion (1976) examined cultured synovial cells for the presence of viruses, in particular retroviruses. Various induction methods were followed by ^{3}H-uridine labelling and also after the synovial cell strains had been co-cultivated with a variety of permissive cell lines from several species. In addition the supernatant fluids were assayed for the presence of RNA-dependent DNA polymerase activity. No evidence was found to indicate the presence of viral information within the synovial fibroblasts.

Immunological evidence of past or persistant infection with viruses

a) Antibody in serum of RA patients

Numerous workers have compared the frequency, or mean titre of viral antibodies in rheumatoid patients with those of control groups. The antibody surveys are listed in Table 1. With

Table 1

Investigations of viral antibodies in sera of RA patients

	Rubella	Measles	Mumps	Herpes	Others	Methods and Comments
Smiley and Casey (1969)	RA = non RA	–	–	RA < non RA	Adenovirus RA = cytomegalo-virus non RA	RF + CF
Kacaki et al (1970)	RA = non RA	–	–	–	–	HAI
Simsarian et al (1970)	RA = non RA (HAI)	RA = non RA (HAI)	RA = noh RA (CF + HAI)	RA < non RA (CF)	Varicella-zoster RA = non RA	RF +
Chandler et al (1971)	RA = N	–	RA = N	RA > N	25 other antigens RA = N	RF + HAI
Vesikari et al (1971)	RA = N (CF)	RA > N	RA = N	RA = N (CF)	Influenza reovirus parainfluenza polio RA = N	RF +
Kalliomaki and Halonen (1972)	–	RA N	–	RA < N	Parainfluenza I cytomegalovirus varicella-zoster RA = N	RF + CF
Laitinen et al (1972)	RA = N	RA > N	RA = N	–	Reovirus influenza parainfluenza RA = N	HAI
Stanford (1972)	–	RA > non RA	RA = non RA	RA > non RA	–	RF –

Investigations of viral antibodies in sera of RA patients

	Rubella	Measles	Mumps	Herpes	Others	Methods and Comments
Stanford and Shirodaria (1973)	–	–	–	RA = N	–	IgM immunofluorescence
Wilkes et al (1973)	RA = non RA	RA = non RA	RA = non RA	RA = non RA	Cytomegalovirus Hepatitis RA = non RA	CF
Patterson et al (1973)	RA > N	–	–	–	–	RA +
Deinhard et al (1974)	RA > N	–	–	–	–	RF +
Phillips et al (1976a)	–	–	–	–	EBV and cytomegalovirus RA = N	RF +
Hart and Marmion (1977)	RA = non RA	–	–	–	–	RF +

Key:
- RF + = No attempt to remove rheumatoid factor
- RF – = Rheumatoid factor removed before serum tests
- N = Control group composed of healthy population
- Non RA = Control group composed of patients with other forms of arthritis
- HAI = Haemagglutination inhibition test
- CF = Complement fixation

TABLE 1: Investigations of viral antibodies in sera of RA patients.

most antigens there was no difference in the antibody titres when sera from RA patients were compared to healthy controls or patients suffering from other forms of arthritis. Complement fixation was found to be inhibited by the presence of the rheumatoid factor, thus artificially lowering viral antibody levels in the rheumatoid group (Kalliomaki and Halonen, 1972; Stanford, Roberts and Connolly, 1971). The situation was reversed when the rheumatoid factor was removed prior to the test (Stanford, 1972; Haukenes, 1974). Two viral antigens, namely Herpes simplex and measles, have shown the consistent trend of higher levels of antibodies in RA patients as compared to healthy controls. However, IgM Herpes antibodies, indicative of a persisting viral stimulus, were not increased as tested by immunofluorescence (Stanford and Shirodaria, 1973).

Rubella antibodies have generally failed to show a difference except in the surveys described by Patterson, Howard and Deinhardt (1973 and pers. com.) and Deinard et al. (1974). The difference in the mean rubella titre in the latter survey was due to a small group of patients who had very high titres but most patients fell into the frequency range of the controls. Small groups of RA patients with unusually high rubella titres have been observed by others (Smith, C., pers. com.)

b) Antibodies in synovial fluid

No statistically significant differences were found between the concentrations of viral antibodies in serum and in the synovial fluids of RA patients. Herpes, mumps and measles were

tested by complement fixation, rubella by haemagglutination inhibition (HAI), all were related to the IgM levels (Stanford, 1974). Measles HAI titres have been found by other workers to be slightly higher in synovial fluid than in serum (Kalliomaki, Halonen and Salmi, 1975). Kalliomaki and his co-workers also noted lower levels of measles antibody in sera and the synovial fluid from patients with low levels of haemolytic complement. This lower antibody level might be due to specific binding to measles antigen as the same phenomenon was not observed with either rubella or influenza antibodies.

A comparison of cryoprecipitates from blood and synovial fluid did not reveal higher concentrations of viral antibodies in synovial fluid. The overall highest titres was to Herpes simplex (Cremer et al., 1974). Synovial membrane eluates were also examined for viral antibodies without, however, detecting any striking amounts (Zvaifler, 1973).

c) Cell-mediated immune response to viral antigens

The in vitro lymphocyte transformation to Herpes simplex was assessed by ^{3}H-thymidine uptake (Smiley and Casey, 1969). One of fourteen batches of RA peripheral blood lymphocytes responded as compared with 8/16 batches from non RA patients. No other systematic investigation has been carried out with RA patients using for example migration inhibition test so extensively applied to the investigations of mycoplasma involvement in RA (Brostoff and Roitt, 1975; Maini et al., 1975). A group of SLE patients was shown to be less responsive than normal individuals to six of

eight virus antigens tested as measured by ^{3}H-thymidine incorporation by lymphoblasts. This hyporesponsiveness was not reversed by washing the SLE lymphocytes in normal AB plasma and some functional defect in cell-mediated immunity was postulated (Wolf and Ziff, 1976).

The failure by this diversity of techniques to detect a cytopathic virus infection or a productive non-cytopathic infection in synovial cells of fibroblasts, together with the lack of immunological evidence of persistent infection with various common viruses, directs attention to a possible virus-cell relationship with partial expression of a virus genome or expression of genome fragments. This stimulated a search for viral antigens, viral enzymes and viral genetic information.

Studies on antigenic and other viral markers

a) rubella virus

Of the known viruses, rubella virus (RV) has aroused a considerable interest for the following reasons: A self-limiting synovitis occasionally arises as a complication of acute or induced rubella infection and the virus can then be recovered from the synovial effusion (Lerman et al., 1971; Weibel et al., 1969). In addition, the latex fixation RF test may become positive (Kacaki, Balduzzi and Vaughan, 1970). The phenomenon of persistent rubella infection has been demonstrated in the congenital rubella syndrome and also from persistent infection in cultured cells in vitro (Maassab, Veronelli and Hennessy, 1964). In animal studies, rubella antigens can be demonstrated in the chondrocytes of the articular cartilage (London et al., 1970) after intravenous

infection of virus.

Evidence has been sought in different ways for the hypothesis that a persistent infection of synovial cells with rubella virus might be the antigenic stimulus in rheumatoid arthritis. Numerous workers have compared rubella antibody levels in sera of rheumatoid patients with healthy controls or patients suffering from other forms of arthritis, but generally failed to show any difference (Table 1) except Patterson, Howard and Deinhardt (1973) and Deinhard et al. (1974). Deinhard et al. (1974) in particular demonstrated a small group of rheumatoid patients with high rubella titres while the remainder of the patients fell within the normal rubella antibody distribution. This might, however, only illustrate that rubella virus infection is protracted in some rheumatoid patients, a phenomenon similar to that observed in juvenile rheumatoid patients. Ogra et al. (1975) demonstrated high and persistent levels of IgM and IgG antibody to vaccine rubella virus and rubella virus antigen in the smears of synovial fluid cells from juvenile RA patients.

The mechanism of chronic cellular infection by rubella virus is not completely understood but is most likely to be persistence of infectious virions or viral RNA as cytoplasmic entities that are transmitted at cell division. Attempts to demonstrate infectious rubella virus in RA synovial fibroblasts have been covered by the conventional virological techniques mentioned previously, as well as by the attempts to demonstrate incorporation of ^{3}H-uridine into RNA produced by synovial fibroblasts with sedimentation characteristics of rubella virions (Grayzel, 1973;

Person, Sharp and Rawls, 1973; Norval and Marmion, 1976). None gave evidence of the presence of vegetative rubella virions in these cells. The recent claims to have demonstrated a RNA-dependent-DNA polymerase associated with a rubella temperature sensitive mutant invokes the possibility of persistent rubella virus in the form of an integrated DNA copy of the RNA viral genome (Sato, Yamada and Yamamoto, 1976, 1977) with only the rubella antigens expressed in the infected cells.

The brief preliminary reports by Patterson, Howard and Deinhardt (1973, 1977) were, therefore, of much interest. These workers measured the release of ^{51}Cr from 6 strains of RA synovial fibroblasts treated with complement and a rubella antiserum from a hyperimmunised rhesus monkey and demonstrated (presumptive) rubella antigens on 4 out of these 6 strains of RA synovial fibroblasts. The controls included a chronically infected cell strain from an infant with the rubella syndrome and other non-RA fibroblasts.

b) measles

Measles virus has been demonstrated to persist in cultured cells *in vitro* (Rustigian, 1966) and *in vivo* in cases of subacute sclerosing pancencephalitis (Norrby, 1973). Antibody to measles virus was found in cerebrospinal fluids from patients with multiple sclerosis at a higher concentration than in peripheral blood (Salmi *et al.*, 1972) and at higher levels compared to other neurological disorders (Haire, Milla and Merrett, 1974) suggesting persisting antigenic stimulation by measles.

There is some evidence of increased titres of antibodies to measles virus in RA (Kalliomaki, Halonen and Salmi, 1975) and an association has been suggested between measles antibodies and lower haemolytic complement levels in synovial fluid. However, peripheral blood lymphocyte from RA and control group responded to the same degree in lymphocyte transformation test when cultured with measles antigen (Panayi, 1976; unpublished observation).

The interest in this virus has been maintained by electron microscopic observations of SLE in which tubular paramyxovirus-like structures are commonly seen (Schumacher, 1970), although these have now been shown to be of cellular origin rather than paramyxoviruses (Schaff, Barry and Grimley, 1973).

No direct study for antigens or attempts to detect specific viral enzyme (neuraminidase) have been carried out.

c) retroviruses

This diverse group of RNA viruses is characterised by an RNA-dependent DNA polymerase (reverse transcriptase) enzyme in the virions (there are various other synonyms - RNA tumour virus, oncornavirus - also leukoviruses, Fenner et al., 1974). One subgenus of the retroviruses comprises the group of C-type viruses, which have been isolated from mammals, birds and reptiles. Mammalian C-type viruses currently fall into three classes based on interspecies cross-reacting antigens (viz: the major internal polypeptide p30, the viral envelope glycoprotein gp 70 and the reverse transcriptase). The classes are (i) woolly monkey virus

(syn. simian sarcoma associated virus) and the gibbon ape virus, (ii) RD-114 and baboon endogenous virus, (iii) the murine, rat, hamster and classical feline viruses (Strand and August, 1975; Todaro et al., 1974; Scolnick et al., 1972). Another retrovirus mentioned in this thesis, the Mason Pfizer mammary tumour virus, belongs to a different subgenus, the B-type viruses, but on the basis of immunological cross-reactivity is related to the group of mammalian endogenous C-type viruses (Stephenson et al., 1976). In this thesis the name retrovirus is used to cover the mammalian C-type viruses and one representative of the B-type group.

For the history and pathology of these viruses, the reader is referred to Gross (1970) and for the experimental and molecular biology, to Gilden (1977).

The association of retroviruses with the systemic lupus-like disease of the New Zealand Black mouse and of hybrids between New Zealand Black and White mice is dealt with in the next section on viruses and the immune system.

The connection of retroviruses with human systemic lupus erythromatosus (SLE) is less well documented. Infective virus has not been isolated (Phillips et al., 1976b), but recent studies suggest that the expression of various cross-reacting antigens is enhanced in human SLE tissues (Strand and August, 1974). It is also claimed that lymphocytes from SLE patients show immunofluorescent staining with an antiserum against a retrovirus produced by a plasmacytoma (SP104 cells) induced in mice during the canine lupus experiments; the staining of SLE lymphocytes was reduced

by previous adsorption of the antiserum with the retrovirus virions (Lewis et al., 1974; Schwartz, 1975). In a slightly different approach, Mellors and Mellors (1975) and Panem et al. (1976) have examined sections of SLE kidney with antisera against various primate, or primate-related, retroviruses and found viral proteins in the immune complex in the glomeruli. Naturally enough, this work stimulated widespread interest but attempts to repeat it by examination of lymphocyte and other tissues (including placenta in which C-type particles may be seen) for infective virus, or viral antigens or nucleotide sequences have been negative (Phillips, 1977). The possible involvement of retroviruses in RA has been the subject of limited investigations. Reverse transcriptase enzyme has been looked for in the synovial membrane homogenates by two groups but not detected (Norval, Ogilvie and Marmion, 1975; Spruance et al., 1975). Co-cultivation of rheumatoid synovial fibroblasts with cell lines permissive for primate retroviruses were negative (Norval and Marmion, 1976). RA lymphocytes and synovial cells other than fibroblasts have not been examined for viral antigens in a way analogous to the observations in SLE, nor have patients been studied for antibody.

Attempts to transmit RA to experimental animals and chick embryos

Warren et al. (1969) reported the development of "arthritis" in the descendents of pregnant mice injected with homogenised synovial membranes from rheumatoid arthritis patients. Others have failed to show any difference in the frequency or the distribution of deformities in mice injected with RA tissue,

and those injected with membrane homogenates from non-RA sources (Ward, Cole and Smith, 1972; Kruit et al., 1973). More recently Crocker et al. (1974b) produced arthritis in the offspring of pregnant, complement deficient (C5) mice injected with rheumatoid synovial membrane homogenates, thus reawakening interest in transmission experiments. The fourth generation of inbred progeny still developed transient swelling and inflammation of their tails and all four paws; recurrent episodes led to persistent deformities of the tails and limbs. Similar lesions were not observed in uninoculated mice or in the progeny of mice injected with OA synovial membranes.

Apart from the experiments with mice, claims have also been made of transmitting RA to other animals including rats (Warren et al., 1972) and chickens (Warren et al., 1971). Warren et al. (1975) went on to characterise some of the properties of this "transmissible" agent. The assay system comprised injection of ten-day-old embryonated hens' eggs which were subsequently scored for arthritis and limb deformities as hatched chicks. The agent had high thermal resistance (60 min at 121°C) and a density of 1.44-1.49 g/l on caesium chloride gradients. The thermal resistance is similar to scrapie agent and the implications of these findings are exciting. The positive results consistently obtained by this one research group unfortunately have not been confirmed by other workers in this field.

Besides the attempted transmission of RA to rodents and birds, one attempt has been made to induce disease in higher mammals.

A small group of healthy baboons was injected with rheumatoid synovial membrane homogenates and synovial fluids, and also with Chang cells containing 'bleb' structures derived from co-cultivation experiments with synovial fibroblasts (Mackay et al., 1974). The baboons were observed for 3 years without any visible changes and then killed for pathological examination. There was no increase in antibodies to a range of common viral antigens apart from one baboon who developed rubella HAI at a titre of 64 during the experiment. The pathological investigations were completely negative (Duthie et al., to be published).

Other observations with animals

Although the dog is reported to develop a condition like RA, this does not appear to have been exploited in RA research. There is an extensive literature on possible similarities between RA and arthritis caused by infection with mycoplasmas, Erysipelothrix insidiosa, or chlamydiae. These conditions are of much interest in their own right but have not proved useful as pointers to organisms that are actually involved in RA and will not be reviewed in this thesis. Similarly, experimental models of arthritis induced in laboratory animals by immunological means (see Dumonde et al., 1977) and of arthritis associated with immune complex lodgement in rabbits (Poole and Coombs, 1977) are of much interest as (secondary) immunological mechanisms, but are not directly related to the question of viruses in RA and so are not reviewed in detail here.

INTRODUCTION

CHAPTER IV

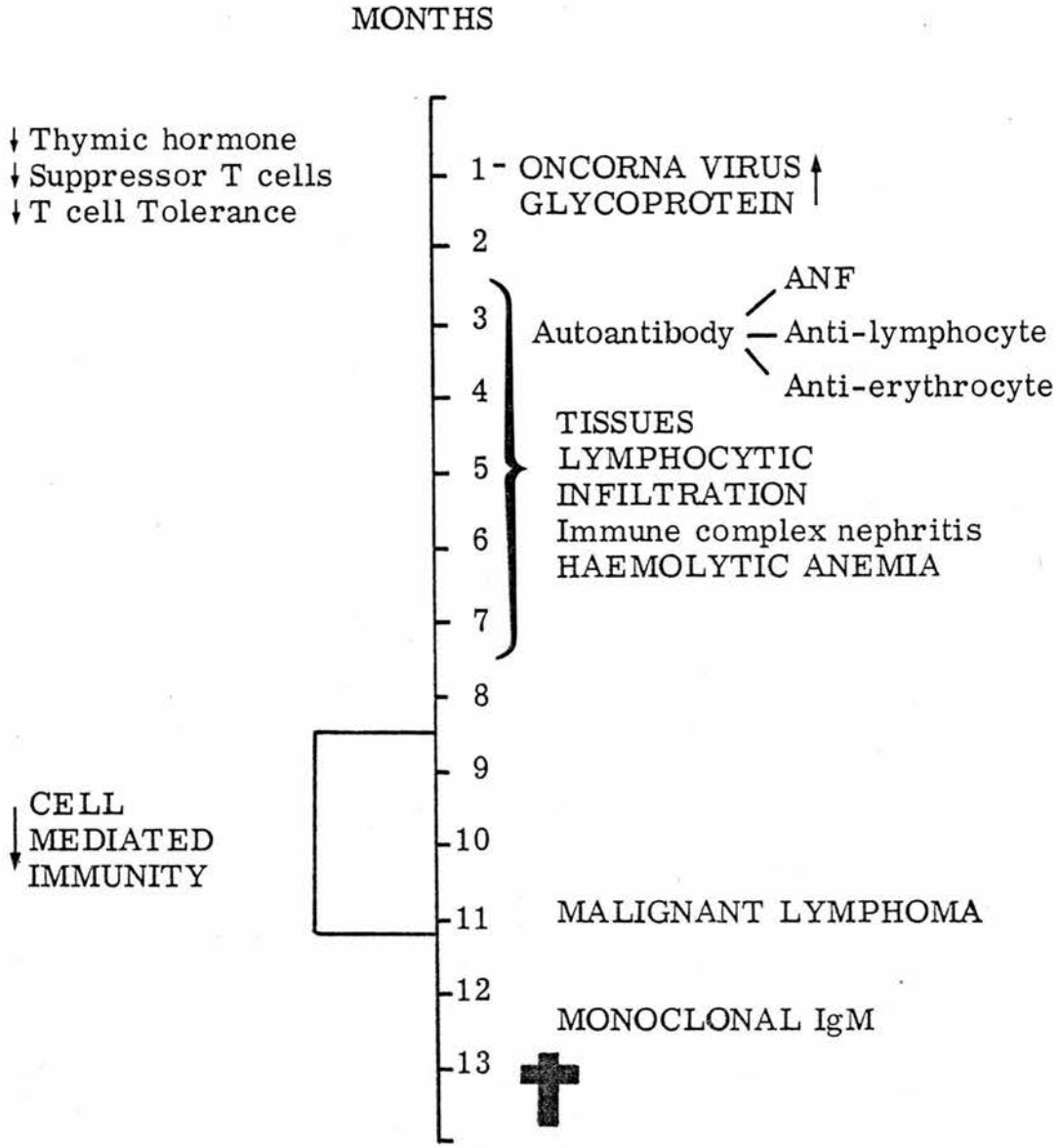
VIRUSES AND THE IMMUNE SYSTEM

Any investigation of viruses in RA has to take account of the findings from the rapidly developing and diversified field of viral immunopathology, mostly related to observations in animals but a few from man.

Consideration may be given either at the level of the whole animal or at that of the interaction of virus with T or B lymphocyte or macrophage.

Certain persistent virus infections (lactic dehydrogenase virus, lymphocytic choriomeningitis virus, various leukemia viruses (retrovirus group)) in mice, and Aleutian disease in mink, appear to be examples of a failure of the T lymphocyte/ macrophage effector mechanisms to eliminate virus-producing cells, while B lymphocytes produce both antiviral antibody and a range of antitissue antibodies (RF, ANF, anti-erythrocyte, etc).

The most striking feature of all the persistent virus infections mentioned above, is the formation of immune complexes of viral antibody and antigen, and antitissue components, that lodge in the glomeruli. In addition, in the retrovirus infections, perhaps also as a reflection of diminished T lymphocyte activity, there may be increased incidence of lymphoid tumours of leukaemia.



NATURAL HISTORY OF 'LUPUS' IN NZB and NZB/NZW MICE. (After TALAL, FYE AND MOUTSOPOULOS, 1976)

Figure 2: Schematic representation of the events in the autoimmune complex disease of the NZB and NZB/NZW mice.

Much attention has been paid to the NZB mouse and its various hybrids as a model of these viral immunopathological processes.

The disease complex in NZB/W hybrids - haemolytic disease with incomplete, anti-erythrocyte antibody, immune complex nephritis, and lymphoma - occurs spontaneously and was thought originally to be inherited. However, cell and bacteria-free filtrates of homogenised cells from enlarged spleen and lymphoma tissues, when injected into Swiss mice, induced Coomb's positive haemolytic disease, and renal glomerular disease in 20% of the recipients (Mellors, 1968; Mellors, 1969; Mellors and Huang, 1966, 1967).

It was subsequently established that the disease is associated with an increase of gp 69/70 antigen of an endogenous retrovirus in the spleen and serum and that the complexes in the glomeruli contained antibody and complement bound to viral antigens and to various tissue components (DNA etc.) (Mellors et al., 1971; Yoshiki et al., 1974). The natural history of the various disease manifestations and changes in immune function is summarised in Figure 2.

The haemolytic anaemia and the immune complex nephritis are observed to occur at different rates in the various hybrids and back crosses and also differ in their associations and in the facility in which they can be transferred adoptively. Thus, only the Coomb's positive haemolytic anaemia can be transferred by B lymphocytes (Heer and Edington, 1974; Purves and Playfair, 1973),

whereas the immune complex nephritis is correlated with the level of expression of the retrovirus antigens in line with the general concept advanced at the start of this section. Crocker et al. (1974) injected the so called Scripps leukemia virus (SLV 60A), originally isolated from lymphoblastoid cell lines established from the peripheral blood of NZB mice into neonatal (BALB/c x NZB) F1 recipients, and induced anti-nuclear antibodies (ANA), immune complex nephritis and thymocytic lymphomas. However, they failed to induce the Coombs-positive haemolytic anaemia. A curious feature was that the concentration of retrovirus antigens remained proportional to the initial dose, thus differing from conventional viral infections, where maximum viral concentration would be reached irrespective of the dose. Cannat and Varet (1973) induced ANA, but not glomerulonephritis by the inoculation of Friend-Moloney-Rauscher (FMR) group of MuLV into (BALB/c x C57/B16) F1 mice. Similar ANA induction was observed in mice with another isolate of MuLV shed by the SP104 cells of mouse plasmacytoma (Schwartz, 1975).

Not all investigators consider that it has been established beyond doubt that the viral infection is a primary cause of the autoimmune disease in the NZB/W model. Denman (1976), for example, suggests that the virus activation is a process secondary to a basic immunoproliferative abnormality or immune deficiency. On the other hand, it could be argued that the virus replication interferes with immune regulation and allows the immunoproliferation. Several observations support this notion: (a) retroviruses can

replicate in lymphoid cells; e.g. the SLV virus was isolated from lymphoblastoid cell line (Lerner et al., 1972) see also review by Dent (1972); (b) the concentration of retrovirus antigen appears to be associated with a proliferation of neonatally infected cells rather than overall infection (Crocker et al., 1974); (c) mouse thymocyte differentiation antigen GIX has been reported to be identical with gp 70 glycoprotein antigen of murine oncornaviruses (Obata et al., 1975).

The most pertinent implication that can be drawn from the NZB/W mouse model, is one of the imbalance between B-cell activity and antibody responses and T-cell activity or cell-mediated immunity which are depressed (Talal, 1970; 1976; Talal et al., 1977) together with the development of auto-antibodies (to ds DNA); another correlate of abnormal T-cell regulatory mechanisms.

In man, Hepatitis B infection is the clearest example of a viral immune complex disease (Reed, Eddleston and Williams, 1974), and has some of the features of the mouse models just discussed. Circulating complexes of HBsAg and IgG have been found in serum (Almeida and Waterson, 1969) and are deposited with complement in the renal glomeruli, arteries or liver, in patterns that vary from patient to patient and in various age groups. Ig has been eluted from complexes in tissue and identified as anti-HBs.

Occasionally arthritis is associated with hepatitis B infection (Onion et al., 1971). The circulating immune complexes are probably responsible, although Schumacher and Gall (1974) produced some evidence of the presence of virus in joint cells. The high

frequency of autoantibodies in HBsAg positive patients is of some interest (Dudley, O'Shea and Sherlock, 1973). In this study 87% of antigen positive patients had smooth muscle antibody at titres unrelated to those of HBsAg. The anti-smooth muscle antibody was predominantly IgM (Farrow et al., 1970) and may, of course, be related to the damage to the hepatocytes. Rheumatoid factor is commoner in patients with chronic aggressive hepatitis or active cirrhosis, and is found less often in patients with acute and chronic persistent hepatitis. Antinuclear factor is not common in HBV infection, although it is present in a small proportion of patients (Vischer, 1970). There is evidence of depressed T lymphocyte function in HBV infection, so the findings with autoantibodies is again significant. The arthritis observed in HBV immune complex disease is transient, and in terms of damage to the joint surface and synovial membrane is clearly different from the production of immune complexes and continuous inflammation in RA. For full comparison of RA and hepatitis B arthritis see Marmion and Mackay (1977); similar production of complexes from an extra-articular site and lodgement in the synovium seems an unlikely mechanism in RA.

Other virally-induced modifications of immune function

The general pattern of diminished T lymphocyte regulation of B lymphocyte function that emerges from the above consideration of animal models may be supplemented by a brief review of infection with two herpes viruses, EB virus and Marek's disease virus, that are known to infect B and T lymphocytes respectively.

The best studied of the two is Epstein-Barr virus infection. The general background is reviewed by Miller (1975), Glaser and Rapp (1976) and Epstein and Achong (1977). B-lymphocytes have a specific receptor for EB virus, which correlates with the presence of complement receptors on the cells (Einhorn et al., 1978); T-cell fractions do not absorb virus. It is now well established that lymphoblastoid cell lines grown from Burkitt's lymphoma, or the peripheral blood cells of persons with acute or past infectious mononucleosis, contain the EB virus genome in multiple copies and in varying degrees of expression; they are of B-cell origin (references in Epstein and Achong, 1977). During infection, a virus coded antigen (LYDNA) is expressed on the membranes of peripheral blood B-lymphocytes. This antigen is the only EB virus-determined antigen to which the patient does not respond by formation of antibodies (Klein, Klein and Levine, 1976; Svedmyr and Jondal, 1975). It is also remarkable that infection of lymphocytes by this virus leads to a 36-fold enhancement of human leucocyte HLA antigen expression in the infected cells (McCune et al., 1975).

In infectious mononucleosis, both B and T-lymphocytes increase in numbers following EBV infection. Atypical lymphocytes appear, some are T-cells and generally are not virocytes (Miller, 1975); the overall reaction appears to be a substantial T lymphocyte blast reaction against virus coded antigens on B cells.

The transient heterophile and auto-antibody production (Davidson and Lee, 1969) is of interest in relation to auto-antibody in RA. The commonest auto-antibodies are to smooth muscle or to

contractile fibres in other tissues including human thyroid (Sutton et al., 1974). Lymphocytotoxins, cold reacting IgM antibodies to erythrocytes, have been detected by others (Mottironi and Terasaki, 1970; Thomas, 1973) as well as antibodies for lymphocyte membranes measured by immunofluorescence (Sutton et al., 1974); specificities are for a subpopulation of T lymphocytes (Thomas, 1973) or for B lymphocytes in the blast phase (Thomas and Phillips, 1973). The presence of antibodies against smooth muscle or lymphocytes does not correlate with other auto-antibodies in the sera; these include, at a lesser frequency, antinuclear antibodies (Kaplan and Tan, 1968) and rheumatoid factor (Carter, 1966). Some antibodies are entirely in the IgM class (Carter, 1966; Thomas, 1973). Their appearance, usually at low titre, seems to coincide with maximal lymphoreticular proliferation. It is suggested that clones of B lymphocytes form these antibodies as a result of infection with EB virus (Sutton et al., 1974). It is a reasonable supposition that the cells containing virus are the ones making the antibody, although this is not quite certain. Studies of lymphoblastoid cell line in serial culture show that they are synthesising immunoglobulins with μ, γ and α heavy chains and κ and λ light chains (Glade and Hirschorn, 1970). The antibody specificities of such immunoglobulins has not been studied extensively but include antibody to untreated and glutaldehyde treated sheep erythrocytes (Joss et al., 1976) and RF in the IgG and IgM classes (Ford and Smiley, 1973, and personal communication).

There is an evidence for the existence of clones of self-reactive human B lymphocytes in normal individuals which can be activated by polyclonal B cell stimulations such as bacterial lipopolysaccharide, phytohaemagglutinin or antibody fragments ($F(ab)_2$) to produce auto-antibodies (Primi et al., 1977). EBV may exert similar mitogenic stimulation of normal B lymphocytes, which would result in the production of auto-antibodies in vivo or the effect may arise from the genetic interaction of the virus and host cell.

The outpouring of lymphocytes and atypical cells in infectious mononucleosis is also associated with depressed cell-mediated immunity (Miller, 1975); diminished skin reactivity to tuberculin, hyporesponsiveness of peripheral lymphocytes to PHA and to allogeneic lymphocytes responses can all be demonstrated. As the T lymphocytes are not infected by EBV, the most likely explanation of the depressed cellular-immunity might be the presence of the anti-lymphocyte antibodies.

In view of the role of various subsets of T lymphocytes in controlling the expression of the B lymphocyte, it would be of much interest to know whether viruses that infect the stem cells, precursors of various mature subsets of T lymphocytes would release B cell activity and allow auto-antibody formation. Virus-mediated effects on graft rejection, delayed hypersensitivity reactions, T cell helper function and antibody formation are documented by Dent (1972). It is clear that measles, rubella, retroviruses, and hepatitis viruses have effects at various maturation levels but these are difficult to pinpoint exactly.

Another model of virus T lymphocyte interaction is that in Marek's Disease (MD), a lymphoma of chickens caused by a Herpes virus. For general reviews, see Nazerian, Lee and Sharma (1976); Payne, Frazier and Powell (1976).

This is a unique system in which both immune effector cells and the target cells are T lymphocytes (Sharma, 1975; Ross, 1977). In MD tumours there is a predominance of thymus-derived lymphocytes (Hudson and Payne, 1973; Rouse, Wells and Warner, 1973) and the establishment from these tumours of a number of cell lines bearing thymus specific and tumour specific antigens (Witter et al., 1975) indicates that thymus-derived cells are neoplastically transformed in MD. In addition to the antigens already mentioned, these cell lines also carry virus-specific antigens that are serologically distinct from the tumour antigens (Ross et al., 1977).

Given the involvement of T lymphocytes, is there decrease of B lymphocyte function? Serum immunoglobulin levels in infected chickens have been examined in some detail by Higgins and Calnek (1975a, b). Twenty-one days after infection, IgG increased to levels about 8 times higher than in control birds and IgM levels were increased two-fold. The antibody specificities of the high levels of IgG are, unfortunately, unknown at present, although some will be, presumably, antiviral. Precipitating and neutralizing antibodies to Marek's Disease virus (MDV) at 7-14 days have been described (Higgins and Calnek, 1975a, b) but do not really explain the bulk of IgG produced later. Auto-antibodies have not been looked for systematically but antibody to myelin is

formed and is perhaps responsible for the demyeliation of the nerve tissues observed in chickens with lymphoma (Ross, pers. comm., to be published).

The activity of T lymphocytes from chickens in various stages of the disease has been also examined in some detail. Lymphoid cells obtained from spleen or tumours of chickens with MD have an impaired *in vitro* proliferative response to PHA as measured by incorporation of ^{3}H-thymidine (Burg *et al.*, 1971; Alm *et al.*, 1972) but peripheral blood lymphocytes show normal response (Ross, pers. comm.).

Although the burst of autoantibody production in infectious mononucleosis is shortlived, and terminated by reassertion of T lymphocyte control over these B cell functions, the model serves to show that a virus-driven synthesis of antitissue antibodies is possible. In extrapolating the findings as a possible model for RA, it might be supposed that 'virus' could turn-on autoantibodies (RF, antilymphocyte) on a more permanent basis, associated, or induced by the persisting viral genes, and that a defect in T cell regulation might allow them to continue, perhaps in an individual with a particular genetic constitution involving cell mediated responses. Interestingly, there appears to be some clonality in the production of the autoantibodies (IgG-rheumatoid factor) within the rheumatoid synovial membrane (Natvig, Munthe and Pahle, 1975). While most tissues contained plasma cells corresponding to all the four IgG subclasses, a very striking clonal appearance of other plasma cells was observed. In certain areas clusters

of IgG3 plasma cells could be seen, while in other areas IgG1 or IgG2 cells predominated. IgG4 was more rarely seen.

OBJECTIVES AND SCOPE OF PRESENT WORK

The foregoing review has been written or revised in the light of knowledge in 1978 but the work described in this thesis began in 1973. At that time, as will be apparent from the review, virological investigations had established, essentially, that a cytocidal infection with a common virus was unlikely as a cause of RA, and there were indications from the serological surveys and limited experimentation with synovial cultures that a persistent, productive non-cytocidal infection was also unlikely. Non-productive viral infection with incomplete gene expression remained a possibility.

Most attention had been concentrated on synovial fibroblasts as (presumed) target cells and less attention had been directed to other cells in the joint - vascular endothelium, stromal cells, cartilage cells and the lymphoid and macrophage elements. Little attention had been paid to the possibility of viral alterations to immune effector cells as a possible basis for the disease. Among the virus groups to be considered as possible 'candidates' as etiological agents, retroviruses had not been studied in any depth.

The work to be described in the sections that follow was undertaken as my contribution to a research programme into the possible role of viruses in the etiology of rheumatoid arthritis, supported by the Nuffield Foundation and based in the Virus Research Laboratory in the Department of Bacteriology at Edinburgh.

The research programme in general covered various approaches to the problem (e.g. broad ones such as serological surveys of RA and non-RA patients for differing or unusual prevalences of antibody to various common viruses) but, as the result of the 'target' hypothesis, then current, it concentrated at first on the examination of synovial cells (synovial 'fibroblasts') for evidence of a non-cytocidal productive virus infection, or of virus-coded or virus-induced neoantigens on these or other cells elsewhere in the synovial membrane or joint.

It was also reasoned that the expression of viral antigens on joint cells should lead to local synthesis of antibody, and/or the binding of circulating antibody. And, consequently, that examination of immunoglobulins eluted from RA synovial membrane, or those synthesised in vitro by membrane explants, for virus-specific antibody, might also provide a useful, indirect, pointer to viral activity.

In this first phase of my work the main emphasis was placed on infection with rubella virus. The general reasons for interest in the arthropathies produced by this virus have been considered earlier but there were, in addition, two events at that time that caught our attention. The first was the clinical observations of a patient (Mrs A) who developed a mild, but typical, rheumatoid polyarthritis after an attack of rubella. (The clinical, virological and serological findings are described under 'Results').

The second was the report of Patterson, Howard and Deinhardt (1973) who claimed - and still claim (Patterson, Howard and Deinhardt, 1977) - that a high proportion (14/19) of RA synovial cell cultures have rubella virus antigens, as judged by ^{51}Cr release in an antibody and complement dependent cytotoxicity test with a rubella antiserum prepared in a rhesus monkey. Fibroblasts from healthy synovial membrane, or from osteoarthroses, showed no cytotoxicity. Investigations in the Virus Research Laboratory (EUMS) and elsewhere had already failed to detect a productive infection with rubella (or other RNA viruses) in RA synovial fibroblasts so that the claims of Patterson et al. (1973), if true, implied an unusual rubella virus-cell relationship; at about that time Sato et al. (1974, 1975) had, in fact, reported an association of reverse transcriptase enzyme with a temperature sensitive mutant of rubella, suggesting an integration (of a cDNA copy) of rubella virus genes in persistently infected cells.

In the circumstances it was clearly necessary to check the observations of Patterson et al. (1973) preferably by simpler and more reproducible serological techniques than complement plus antibody cytotoxicity and ^{51}Cr release. To this end rubella antisera were prepared and synovial cells examined by immunofluorescence and a specially adapted solid-phase radio-immunoassay. The study of cultured synovial fibroblasts was extended, by the immunoperoxidase technique, to sections of RA synovial membrane and articular cartilage, with the same rubella antisera, so as to examine other cell types in the joint. Lymphocytes were concentrated from the synovial fluid and

examined for rubella membrane antigens. Eluates of immunoglobulin, and radiolabelled immunoglobulin from synovial explants, were examined for rubella and other viral antibody.

In the second phase of my work, in view of the emerging evidence from many different directions that synovial fibroblasts do not appear to have virus-coded, or non-viral neoantigens, attention was transferred to the hypothesis of disordered immunoregulation in RA. A search was made for viral genes in lymphocytes, with particular reference to retroviruses, in view of the involvement of these viruses, as already reviewed, in various autoimmune diseases in animals and because of preliminary evidence of their association with human SLE.

In this phase of the work, collaborative attempts were made to activate and isolate retrovirus from lymphocytes by fusion with permissive cells and examination for reverse transcriptase and virion production. In addition, in personal work, sera from RA patients and controls, and synovial fluids or eluates from RA and other synovial membranes, were examined for retrovirus antibody and RA synovial and peripheral blood lymphocytes were tested for retrovirus antigens. In the absence of generally accepted 'human' retrovirus isolates for antiserum production, use was made of the RD-114 virus (endogenous retrovirus of cats) and Simian sarcoma virus, because (a) protein antigens cross-reacting with a (hypothetical) human retrovirus are likely to be represented in these primate or primate related viruses, by analogy with the interspecies antigens in other mammalian

retroviruses and (b) the isolates already reported as (apparently) coming from human sources have been related to these two viruses. Antisera against other mammalian and avian retrovirus proteins were obtained from the National Cancer Institute. In this study, aimed at the detection of viral antigens in the absence of complete virions or other viral gene products particular care had to be exercised to avoid spurious results due to non-viral cross reacting haptens in the cell culture used to prepare viral antigens for raising antisera, and human tissues.

MATERIALS AND METHODS

TISSUE CULTURE METHODS

Cell cultures

(a) LLC-MK_2 cell line (CCL7, American Cell Type Collection) derived from kidney of adult rhesus monkeys, was propagated in 199 medium (Wellcome) supplemented with 1% horse serum. Later the cells were adapted for the growth of rubella virus by culturing them for 2 passages in 199 medium with 2% heat-inactivated (56°C, 20 min) foetal calf serum (IFCS). These cultures were then chronically infected with HPV-77 and Thomas strains of rubella.

(b) Human foetal fibroblasts: Foetal lungs were chopped into small pieces with scissors and scalpel, placed in 0.25% trypsin in Dulbecco's solution and agitated for one hour in a 37°C shaking water bath (Hearson). The free cells were removed and spun at 200g for 10 min. The cellular pellet was resuspended in Eagle's complete medium (1959 modification) (EE medium) supplemented with 10% FCS and seeded at a concentration of 2-4 x 10^5 cells/ml to establish a primary strain of human foetal lung fibroblasts. Dispersed human foetal skin cells, a kind gift from Dr Heather Cubie, were stored in liquid nitrogen in EE medium containing 10% dimethylsulphoxide (D.M.S.O.). For use, the cells were taken out of liquid nitrogen, spun at 1,000 rpm to remove D.M.S.O. and

resuspended in growth medium.

These human foetal cells were used to absorb various antisera (see immunofluorescence and radioimmunoassay).

(c) All other cells used for cell culture were in current use in the Virus Diagnostic Laboratories. They included three lines of epithelial cells: Vero cells (a continuous line of African green monkey (Cerocopithecus aethiops) kidney); Hep2 cells (human, transformed cells originally derived from a nasal epithelioma of a 56-year-old male), RK_{13} cells (rabbit, transformed kidney cell line), and one line of fibroblast cells BHK-21 (a continuous cell line of Golden hamster (Mesocricetus auratus) kidney).

All cell lines, except RK_{13} cells, were grown in EE media containing 10% calf serum, 100 i.u./ml penicillin and 100 mg/ml streptomycin. They were checked routinely for mycoplasma contamination and remained free throughout the study. RK_{13} cells were grown in 199 medium with the same concentration of serum and antibiotics.

Viruses

(a) Rubella. The HPV-77 rubella virus vaccine strain and "Thomas", a recent isolate of a wild strain, were kindly supplied by Drs Gould and Freestone of the Wellcome Research Laboratories (Beckenham).

These strains were grown in RK_{13} cells in which they produced a definite cytopathic effect. To obtain high titres of virus, strains were propagated in Vero cells. A large roller Winchester bottle (2.5 l) was seeded with 10^7 cells and allowed to grow for 2-3 days in EE medium supplemented with 10% calf serum. A 20 ml inoculum of rubella virus was allowed to adsorb onto a washed monolayer of cells. The medium was changed to EE medium supplemented with 2% CS and the infected cells were rolled for 3 days. The harvested rubella virus was stored at -70°C with bovine serum albumin or calf serum added to the concentration of 10%. It was found that storage of the rubella virus considerably reduced its titre; a drop in titre from 10^4 to 10^1 was observed after storage at -70°C after 4 weeks. For this reason the stock virus was always passed through a roller culture (100 ml bottle) of BHK-21 cells before being used in radioimmunoassay and in indirect immunofluorescence experiments, and through Vero cells prior to the inoculation of the large rolling culture for labelling of rubella virions.

To label the rubella virus with ^{3}H-uridine, the medium was changed to 40 ml EE medium with 1% CS and containing 0.1 mCi of ^{3}H-uridine (25 uCi/ml). The infected cells were grown in this medium for 24 hours. The supernatant fluid was removed and spun at 10,000 rpm (max. 12,000 g) in a SS-34 rotor of a Sorvall centrifuge (RC2-B Automatic Superspeed Refrigerated

Centrifuge, I. Sorvall Inc., Norwalk, U.S.A.). The clarified fluid was treated with an equal volume of saturated ammonium sulphate (4°C), which was added slowly with a continuous shaking. After 30 min at 4°C the ammonium sulphate precipitate was removed by spinning at 10,000 rpm (SS-34, Sorvall) and resuspended in 1 ml of Tris-EDTA buffer (0.01M, pH 7.2). The dissolved precipitate was stored in -70°C until analysed on sucrose density gradients.

(b) Measles. The lyophilised Schwartz measles vaccine strain (kindly donated by Dr J. McWilliam, Environmental Health Department, Edinburgh) was resuspended in 1 ml of distilled water, further diluted 1/10 in EE medium, containing no serum, and layered over a semi-confluent monolayer of Vero cells in a 100 ml medical "flat". Large refractile stellate cells, that were absent in the control, uninoculated cultures, were seen 96 hours after inoculation. The supernatant fluid from the infected culture was titrated on flying coverslips of Vero cells. For the titration glutamine was omitted from the EE medium to induce the formation of multinucleate giant cells. The flying coverslips were stained by Giemsa's method (Cubie, 1972) and the titre of the measles virus in the fluid was taken to be the greatest dilution at which syncytia were seen. Virus was stored in 10 ml amounts at -70°C.

To radiolabel the measles virus, it was propagated in a roller culture (2.5 l) of Vero cells for 24 hours at which time

EE medium, supplemented with 1% CS and 25 uCi/ml (5-^{3}H)-uridine was added. Incubation continued for a further 24 hours. The supernatant was freed of cellular debris and precipitated with ammonium sulphate by the same method as described for the rubella virus. The labelled measles virus was used for the examination of the locally synthesised immunoglobulins by the rheumatoid synovial membranes.

(c) Adenovirus type 5 (coding H1526; 25.2.70) was obtained from Dr W. Russell (Mill Hill, London) and propagated in Hep2 cells grown on EE medium with 2% calf serum. A volume of 0.2 mls of CsCl gradient purified adenovirus was diluted to 10 mls with EE medium and absorbed onto a sheet of Hep2 cells in a roller bottle (2.5 l) for 1 hour at 37°C. The excess inoculum was removed and the cell monolayer was washed twice with EE medium to remove any traces of CsCl and growth medium was replaced. After 24 hours the growth medium was replaced with 40 mls of EE medium containing 1% FCS and 0.1 mCi of (methyl-^{3}H)-thymidine and incubation was continued for 24 hours. A cytopathic effect in the cultures of Hep2 cells was observed within the first 24 hours of inoculation with virus and consisted of increased granulocity of the monolayers and the formation of cell clusters. Very few cells remained attached after a further 24 hours. In stationary culture, a similar effect took 5 days to appear. Uninfected Hep2 cells were incubated with ^{3}H-thymidine to act as controls.

The labelled supernatant fluids were freed of cellular debris and concentration by the ammonium sulphate precipitation

method described above.

Cell lines with retroviruses

Feline embryonic amnion cells, chronically infected with feline leukemia virus, and uninfected control cells, were kindly supplied by Dr O. Jarrett (University of Glasgow, Veterinary School). The chronically infected amnion cells (FeLV), and the uninfected control (FEA) were split once a week (EE medium, supplemented with 10% FCS). To radiolabel the virus, the cells from a Roux bottle were seeded into a roller culture bottle (2.5 l) and allowed to grow for 24 hours. 0.1 mCi of (5-^{3}H)-uridine in 30 mls of EE medium with 1% FCS replaced the growth medium for the next 24 hours. The supernatant fluids from FeLV infected and control cultures were processed as described previously and stored at -70°C.

RD-114 virus infected RD cells [human rhabdosarcoma cell line, originally isolated by McAllister et al. (1971) and described by McAllister et al. (1972)] were kindly donated by Dr N. Teich, Imperial Cancer Research Fund Laboratories, London; uninfected control RD cells were obtained from Dr O. Jarrett.

RD-114 RD cells and RD cells were split twice a week (EE medium with 5% FCS). To adapt the RD-114 virus to RK_{13} cells as a preliminary to the production of antigen to immunise rabbits, UV-irradiated RD-114 infected RD-cells (Rowe, Pugh and Hartley, 1970) were co-cultivated with DEAE Dextran (25 ug/ml) treated rabbit kidney RK_{13} cells. The resulting cultures -

control RK_{13} and RD-114 RK_{13} - were grown in 199 medium supplemented with 5% normal rabbit serum. The continuous production of RD-114 virions, which appeared 6 weeks after co-cultivation, was checked by labelling with (5-^{3}H)-uridine and by reverse transcriptase assay (Norval and Marmion, 1976). The possibility that the RK_{13} cells had been overgrown by RD-114 infected RD cells was excluded by testing for the lactic dehydrogenase isoenzymes (Seck, Wayne and Desmet, 1970). The isoenzyme patterns were those of the RK_{13} cells, and not those of the human rhabdosarcoma cell line.

Simian sarcoma-associated helper virus (SSAV) kindly donated by Dr N. Teich, was propagated in a rat kidney cell line originally transformed by Kirstein strain murine sarcoma virus (KNRK). KNRK cells do not produce infectious particles (Stephenson, Scolnick and Aaronson, 1972). The SSAV infected KNRK cell line was called KW23.

Simian sarcoma-associated helper virus producing cell line (KW23) and control rat kidney (KNRK) were also split twice a week (EE medium, supplemented with 5% FCS). For the preparation of antigen to immunise rabbits the cell lines were propagated in medium with normal rabbit serum (5%).

Clinical specimens

The supply and prompt delivery of fresh synovial membrane specimens was greatly aided by Dr M.M. Ogilvie and later by Mrs Hislop, S.R.N. The latter also abstracted the details of diagnosis and clinical state of the patients.

(a) Synovial membranes. Synovial tissues were obtained from patients undergoing synovectomy in the Princess Margaret Rose Hospital (PMRH), Edinburgh. Large pieces of synovial membranes were collected in sterile Hanks' balanced salt solution. Tissue was dissected in the laboratory and only the membranous parts were used, without fatty or collagenous tissue. Fibroblasts from synovial membranes were obtained by overnight trypsinisation of synovial tissue (0.25% trypsin in Dulbecco's solution) at 37°C. The cells were dispersed using a broad-ended pasteur pipette and the cell suspension removed, centrifuged and resuspended at a concentration of 2-4 x 10^5/ml in EE medium with 10% FCS. Depending on the yield of the cells, they were seeded into medical "flats" (10 mls volumes) or Roux bottles (100 mls). As trypsinisation gave variable yields of cells, with some synovial membranes being almost completely resistant to enzymatic dissociation, the method used by Dr Carol Smith (pers. comm.) was also used. Small pieces of synovial tissue (2-3 mm) were carefully placed in plastic Petri dishes (15 mm x 60 mm, tissue culture grade, Falcon plastics) about 10 mm apart in a small amount (1.5 - 2 ml) of EE medium with 10% FCS; the small pieces were half covered by the medium. The Petri dishes were then held in an incubator (Heinicke Company, U.S.A.) with a continuous supply of 5% CO_2 + 95% air mixture saturated with water at 37°C. The Petri dishes were left undisturbed for 3-4 days to allow the fragments of tissue to attach to the plastic and the medium was changed carefully twice a week. In 2-3 weeks there was sufficient outgrowth of

fibroblastic cells to remove with trypsin and establish a cell strain.

(b) Synovial fluid specimens. Synovial taps were done on patients at the outpatient clinic of the Rheumatic Unit of the Northern General Hospital (NGH), Edinburgh, with the co-operation of the medical staff. After withdrawal, the fluids were placed in plastic containers and promptly delivered to our laboratory. Fibroblasts from synovial fluids were established by mixing equal volumes of fluid and EE medium without serum. After 5 days the fluid was replaced by EE medium, supplemented with 10% FCS. Some of these primary cultures were also incubated with sodium aurothiomalate 10 ug/ml (Mycocrisin, 45% metallic gold, May & Baker Ltd., Dagenham, England) and hydrocortisone, 36 ug/ml (Sigma London Chemical Co. Ltd., England). Sodium aurothiomalate is concentrated within phagocytic cells and inhibits their lysosomal enzyme activity (Persellin and Ziff, 1966); it has also been shown to inhibit the cytotoxic and anti-tumour cell activity of macrophages (McBride, Tuach and Marmion, 1975; Ghaffar, McBride and Cullen, 1976). With these inhibitors it was thought that synovial fibroblasts destined for destruction by "activated" macrophages might be preserved for examination. In addition, glucocorticosteroids are reported to increase proliferation of adult human fibroblasts in vitro and reduce the rate of hyaluronic acid formation (Castor, 1965).

All monolayers of fibroblasts were propagated by trypsinisation.

Small volumes of cells in early passage from membrane or fluid were preserved in EE growth medium containing 10% dimethylsulphoxide (D.M.S.O., Spectroscopy grade, BDH Chemicals Ltd., England). Two million cells in 1 ml in an ampoule were gradually cooled by placing at -70°C in a tightly packed polystyrene container, transferred after 24 hours to the gaseous phase of liquid nitrogen and stored until required.

Some of the features of these fibroblast cultures, marked A to N, and in particular their examination for the presence of leukoviruses, are described elsewhere (Norval and Marmion, 1976). Additional fibroblasts used in this study are labelled II-XXI. All cultures of synovial fibroblasts and human diploid cells were mycoplasma free except for culture 'A' from which Mycoplasma orale was cultured.

Synovial fluid and membrane lymphocytes: Synovial fluids were treated with 80 iu/ml of hyaluronidase (bovine testicular type I, Sigma) for 30 min at 37°C before separation of mononuclear cells on Ficoll-Triosil gradients (Harris, 1970). The lymphocyte-rich fraction was washed twice in Hanks' salt solution and incubated in 199 medium containing 100 ug/ml of streptomycin and 100 iu/ml of penicillin supplemented with 20% pooled human serum for 18 hours (lymphocytes). Half of synovial fluid mononuclear cells from each specimen were trypsinised using the method of Wangel and Klockars (1977) (trypsinised and cultured lymphocytes). To carry out the cytotoxicity tests, the lymphocyte-rich fractions were suspended in 199 medium supplemented with 10% inactivated

pooled human serum (IHS). To obtain lymphocytes from synovial membranes the method followed was that described by Abrahamsen et al. (1975). The only modification was the medium used, 199 medium supplemented with 10% heat-inactivated IHS.

(c) Blood specimens were collected by Dr M.M. Ogilvie or Mrs Hislop from patients undergoing synovectomy at P.M.R. Hospital or from the patients visiting the outpatient clinic at the N.G.H. When serum was required the blood was allowed to clot at 4°C overnight, spun at 900 g and the separated serum stored at -20°C. In early attempts to isolate the lymphocytes, blood was mixed with heparin (10 u/ml, Evans Medical Ltd., Liverpool) allowed to stand, the buffy coat harvested and the mononuclear cells separated on Trisol-Ficoll (Harris, 1970). Later, blood was defibrinated to remove platelets from the lymphocyte-rich fractions. Sterile glass beads (4 mm in diameter; 10 beads/ 10 mls of blood) were added to a fresh blood specimen and the container gently rolled till the clotting occurred round the beads, thus trapping the majority of the platelets and leaving the mononuclear cells available for further separation by the Trisol-Ficoll method.

Organ cultures

(a) Synovial membranes. Synovial tissue was cultured by modifications of the organ culture methods of Smiley, Sachs and Ziff (1968), Van Furth (1971) and Herman et al. (1971). Synovial membranes from patients with different diagnoses, but mainly classical and definite rheumatoid arthritis were trimmed to remove

fat and coarse fascia, cut into small pieces 3-4 mm in size and washed once in EE medium without serum. Three ml of EE medium, with only a tenth of the usual amount of amino acids, and with no lysine and iso-leucine, were added per gram of synovial tissue. The medium was supplemented with 5% human serum and L-(U-^{14}C) lysine monohydrochloride and L-(U-^{14}C) isoleucine (50 uCi/ml; The Radiochemical Centre, Amersham) were added at a concentration of 3 uCi/ml of EE media.

The cultures of synovial membrane fragments were incubated in autoclavable polycarbonate tubes and were continually supplied with CO_2 - O_2 mixture (5% - 95%) bubbled through a capillary tube in the medium. The rate of oxygen-carbon dioxide mixture flow was regulated with a GAP meter (GA Platon Ltd.) and was set at 5 cc/min. The incubation period at 37°C varied from 6-24 hours.

At the end of the incubation period the tissue suspension was frozen and thawed once, spun at 18,000 g for 20 min. (Sorvall Superspeed RC 2 - B Centrifuge, SS - 34 rotor). The supernatant fluid was then transferred to smaller tubes and centrifuged at 105,000 g for 1 hour (Beckmann Centrifuge, SW 65 rotor) to remove the insoluble proteins. Unincorporated radioactive amino acids were removed by dialysis against 0.01 M phosphate buffer (pH 7.0) for 72 hours. The amount of radioactive amino acid incorporated into protein was measured at this stage by precipitating a portion with 10% trichloroacetic acid and counting; the remaining dialyzed supernatant fluid was concentrated by lyophilization and stored at

-20°C until required for further experiments. When required the freeze dried immunoglobulins were re-suspended in distilled water to half of the original volume.

(b) Rabbit spleen and auxiliary lymph nodes. Two New Zealand White rabbits were inoculated with a 100x polyethyleneglycol concentrated supernatant fluid from rubella infected RK_{13} cells grown in 199 medium supplemented with 5% rabbit serum. The rabbits were injected with 5.0 ml subcutaneously and 1.0 ml intravenously on the first inoculation. This was followed by 1.0 ml intravenously one week later. The spleen and cervical lymph nodes were removed from the first rabbit two days after the second intravenous injection. The lymph nodes were freed of fat and, together with the spleen, were chopped to small pieces, washed three times in EE medium and suspended in low amino acid EE medium as with the synovial membrane cultures. The culture was then supplemented with 5% rabbit serum and the radioactive ^{14}C-labelled amino acids, incubated with aeration, and the supernatant fluid processed as described above.

The second New Zealand White rabbit was inoculated with an additional 1 ml intravenously one week later and two days after this third inoculation the spleen and cervical lymph nodes were removed and dealt with as described above.

SEROLOGICAL METHODS

Source and preparation of antisera

(a) Retroviral antisera used in preliminary experiments were kindly supplied by Dr J. Gruber (National Cancer Institute, Bethesda). Two batches comprised the following antisera, all prepared in goats: RD-114 (Tween-ether disrupted (TED) virions and part purified p28 protein); woolly monkey fibrosarcoma SSAV1 (TED virions); Feline leukemia (p12 and p28); Mason-Pfizer mammary tumour virus (MPMV) (p12 and p28); baboon endogenous virus (TED virion and p28).

(b) Rubella and retrovirus antisera - immunisation of rabbits

Antisera to HPV-77 rubella were produced by multiple injections of 10 times concentrated supernatant (polyethylene glycol) from RK_{13} culture (199 medium supplemented with 5% rabbit serum free of rubella antibody) into New Zealand White rabbits. Initially, 5 ml of fluid was given subcutaneously and 1 ml intravenously, followed by 4-weekly injections of 1 ml intravenously (Plotkin, 1969). Two weeks after the last inoculation the rabbits were bled and their sera tested by the haemagglutination-inhibition test (HAI) for rubella antibodies; this schedule was repeated until their sera had HAI titres of 1024 - 2048.

Anti-RD-114 and anti-SSAV sera were produced in rabbits using Tween-ether disrupted, sucrose gradient purified viral particles (Bronson, Elliot and Ritz, 1976) grown in RK_{13} and KNRK cells, respectively ("anti-viral" sera). Simultaneously, as a control sera, other rabbits were injected with similar purified fractions from uninfected RK_{13} or KNRK cells ("anti-cell" sera).

For immunisation 0.2 mg of viral protein was mixed with incomplete Freund's adjuvant and injected subcutaneously at weekly intervals (4 times). The antibody titre was further boosted with alum (Al_2O_3, Seravac Ltd.) precipitated antigen given intravenously (twice). Both anti-viral sera were used when a titre of 320 was reached as measured by immunofluorescence on acetone fixed coverslips of RD-114, RD and KW_{23} cells. (The anti-SSAV serum showed specific viral antibody at a dilution of 1/320 only after three absorptions with 0.1 mg of acetone-dried rat liver powder/ml at a serum dilution of 1/80). Anti-RD-114 and control ('anticell') sera are designated R35/RD-114 RK_{13}/6-29.8.77 and R33/RK_{13}/6-29.8.77; anti-SSAV and control sera are R42/KW23/4-9.12.77 and R40/KNRK/4-9.12.77 respectively. No cross-reaction was observed between uninfected RD-cells with either the anti-RD-114 or the control serum. To absorb a weak cross-reaction of anti-SSAV serum with human cells (NC-37 human lymphoblastoid cell line) the

sera were treated with a mixture of human and sheep red blood cells as described above. The rabbit sera were used at 1/40 dilution in attempts to detect retrovirus antigens on synovial fluid (SFL) and peripheral blood lymphocytes (PBL).

(c) Preparation of human and other serum specimens for radioimmunoassay and immunofluorescence

All sera, including positive rabbit control sera, were absorbed with human and sheep red blood cells and acetone-dried human liver powder. One ml quantities of a 1/10 dilution of sera in PBS containing 0.1% NaN_3 were mixed with 0.5 ml of equal mixtures of packed sheep and human erythrocytes. 0.5 ml of 50% human liver suspension was also added. The mixtures were left overnight at $4^{o}C$. Next day they were centrifuged at 100,000 g for 1 hour to remove immune complexes. The sera were taken to be at 1/20 dilution. If the sera were to be absorbed with heat-aggregated IgG (Cohn fraction II) to remove rheumatoid factor, this was done before diluting them to 1/10. Animal sera were treated in a similar fashion for radioimmunoassay but only the rabbit anti-SSAV and the corresponding control ('anti cell') serum required adsorption for immunofluorescence tests.

(d) Elution of antibodies. For low pH elution the method of McCormick et al. (1971) was used. The small pieces of synovial membrane specimens were washed ten times in PBS. The last wash was checked for absorbance at 280 nm and if negative, 10 mls of glycine buffer (0.15M, pH 2.5) were added per gram of wet tissue and the mixture stirred at 4°C for 2 hours. The synovial membrane acid eluate was centrifuged immediately at 100,000 g for 1 hour to remove any dissociated antigen. The pH was adjusted back to 7.2 with 0.2M sodium hydroxide back and the eluate was dialysed for 72 hours against PBS (pH 7.2). The eluates were concentrated to 1/10th of the original volume by freeze-drying or dialysis against polyethylene glycol 20M (BDH).

Later a high salt concentration elution was tried as this causes less damage to the Ig molecule than does low pH treatment (Edgington, 1971). For this method the tissue was suspended in an equal volume of 3M NaSCN (De Saussure and Dandliker, 1969) and stirred for 60 min at room temperature. The centrifugation and the concentration steps of the high salt concentration eluates followed those already described above.

The eluates from cultured and labelled synovial membranes were tested by radioimmunoelectrophoresis while eluates from fresh synovial membranes were tested by haemagglutination-

inhibition tests and indirect immunofluorescence for antibodies to viruses.

Source and preparation of conjugates

(a) Immunofluorescence

Fluorescein isothiocyanate tagged antisera were anti-human IgG, anti-human IgM and anti-rabbit Ig (Wellcome Laboratories, Beckenham, Kent). Fluorescent anti-goat IgG was obtained from Nordic Diagnostic Laboratories. The lyophilised antisera were re-suspended in distilled water in accordance with the maker's instructions, diluted in PBS containing 2% FCS as appropriate (see below) and stored in small volumes at -20°C, thawed only once.

For indirect immunofluorescence the conjugated sheep anti-rabbit immunoglobulin was diluted to 1/10 in 20% beef brain suspension to reduce non-specific staining. The beef brain, taken from a beast slaughtered for kosher beef, was kindly donated by Mr J. Norval. It was homogenised in PBS (pH 7.2) for 2 min to form a thick suspension 20% w/v and stored at -20°C. For membrane immunofluorescence the sheep anti-rabbit conjugate was resuspended in PBS, containing 2% FCS, as the thick brain suspension would have interfered with this technique.

The anti-human IgG and IgM conjugates were first titrated on acetone fixed cells (mainly human lung fibroblasts or BHK-21) sensitised with an optimal dilution of human serum possessing anti-nuclear factor activity (ANF). That dilution giving the brightest ring of immunofluorescence round the cell nuclei was used for further studies. The conjugates were stored in small volumes at this dilution in 20% beef brain at -20°C.

Rabbit anti-goat IgG, fluorescein tagged, was titred on colonies of Mycoplasma fermentans, using an optimal dilution of goat anti-Mycoplasma fermentans serum (Robbin Lab., Chappel Hill, N.C., U.S.A.) and double dilutions of the anti-goat conjugate. The dilution giving the strongest immunofluorescence was usually 1/8 and this dilution was used throughout the lymphocyte experiments.

Mycoplasma fermentans colonies were grown in PPLO medium (Hayward, N. 1975) for 3-4 days. The method of Gould (1975) for preparation of mycoplasma for microscopic study was used. Small blocks of the solid medium containing discrete mycoplasma colonies were cut out and turned face down onto microscope slides (25 mm x 75 mm). The slide with the blocks was lowered into water at 80° till the agar block turned opaque and melted off, leaving the colonies on the slide. The slides with colonies were stored at -20°C until required for the titration.

(b) Radiolabelling of reagents for use in radioimmunoassay

Goat anti-rabbit immunoglobulin (Nordic Diagnostic Laboratories)

(GAR/Ig) was used as starting material to prepare purified ^{125}I-labelled IgG with anti-rabbit Ig specificity.

Initially, 1 ml of the goat anti-rabbit serum was precipitated with 0.5 mls saturated ammonium sulphate at room temperature. The saturated ammonium sulphate was added drop by drop with continuous stirring over a period of 30 min. The precipitate was spun down at 5,000 rpm and re-dissolved in borate buffer (0.01M, pH =8.2) to the original volume. The precipitation was repeated and then the re-dissolved precipitate was dialysed overnight against borate buffered saline. Protein concentration was measured by the method of Lowry et al. (1951) using a bovine serum albumin solution as a standard. Later, the IgG was separated by the method of Reif (1969). 10 g dry weight of DEAE-Cellulose DE52 (Whatman: preswollen and pre-cycled) was stirred in 0.01M phosphate buffer, pH 8.0, to a volume of 55 ml. The pH was adjusted 8.0 $\pm$ 0.1 with 1N HCl. Sediment was allowed to form and the supernatant was decanted. The DEAE cellulose was resuspended in the same buffer and the settling cycle was repeated twice more. The sediment was sucked dry in a Buchner funnel, which contained two layers of Whatman's No. 1 filter paper, 5.6 g of wet weight (equivalent to 1 g of dry weight) was used for 1 ml of the goat serum. The 1 ml of serum was diluted with 3 mls of distilled water to reduce its ionic strength and added to the pre-cycled DEAE-cellulose. The mixture was stirred for 1 hour at $4^{\circ}C$, and then poured into the Buchner funnel and sucked dry. The cellulose was then washed rapidly three times

with phosphate buffer (0.01M, pH 8.0), the supernatant fluid and washings were collected and dialysed against 0.3mM phosphate buffer (pH 8.0). The resulting IgG rich solution was concentrated by lyophilisation (G/Ig G/AR/Ig).

During this procedure the anti-rabbit Ig specificity was monitored by measuring the haemagglutination titres against rabbit-serum-coated, glutaraldehyde-fixed sheep erythrocytes. For coating (Avrameas et al., 1969), fresh sheep erythrocytes were washed three times with saline or until the supernatant fluid was clear. 0.5 ml of packed sheep erythrocytes was mixed with 10 ml of Dulbecco's solution (containing calcium and magnesium). 1 ml of 2.5% of glutaraldehyde (Fluka AG, Buchs SG, Switzerland) diluted in Dulbecco's was added over a period of 30 min with continuous stirring. 1 ml of normal rabbit serum was then added and the mixture was stirred for a further hour at room temperature. The serum coated cells were washed three times with physiological saline and resuspended in saline to a final concentration of 10^8/ml. 0.025 ml of this suspension was added to each of the doubling dilutions of anti-rabbit serum in microtiter plates ('v' shape). The haemagglutination (HA) titre is the reciprocal value of the last dilution showing full agglutination of the coated sheep blood cells. GAR/Ig was found to have a titre of 12,000 HA units/mg of protein, whereas the ammonium sulphate purified G/IgG/AR/Ig had a titre of 4,000 HA units/mg and the DEAE-cellulose purified G/IgG/AR/Ig 7,000 HA units/mg. The lyophilised DEAE-cellulose purified goat IgG was therefore chosen for the labelling

with iodine -125 (100 mCi/ml, protein iodination grade, The Radiochemical Centre, Amersham).

The protein labelling with I-125 by the chloramine T method was carried out as outlined by Hunter (1974). 1 mCi of $Na^{125}I$ in 0.01 ml was mixed with 0.01 ml of phosphate buffer (0.5M; pH 7.5) and 100 ug of protein (G/IgG/AR/Ig) also in a volume of 0.01 ml. 50 ug of chloramine-T in 0.01 ml was added and the mixture was shaken vigorously. Immediately, 240 ug of sodium metabisulphate in 0.86 ml and 0.1 mg of KI in 0.1 ml of distilled water were added to stop any further iodination of proteins. To preserve the antibody activity 10 mg of bovine serum albumin was added in 0.1 ml of saline. The solution was dialysed against PBS (pH 7.2) with numerous changes of buffer for two days, or until the trichloroacetic acid (TCA) precipitate count was 98-99% of the total. The ^{125}I conjugate was diluted in PBS, supplemented with 2% calf serum (CS) to contain 60,000 counts per 100 sec. (c/100s) in 0.1 ml. A new batch of ^{125}I conjugate was prepared monthly.

To test antibodies in human sera reactive with retrovirus antigens goat anti-human IgG (kindly donated by Dr C. Burrell) was prepared in an identical manner to that already described for goat anti-rabbit IgG..

Techniques for examination of synovial and other samples

(a) Immunofluorescence. The indirect immunofluorescence technique was modified from the methods described by Schmidt

et al. (1966) and by Lennette et al. (1967). Semi-confluent monolayers of cells were grown on coverslips (6 mm x 22 mm), previously boiled in distilled water before being sterilised in a hot air oven at 160°C for 1 hour. The coverslips were seeded with 2×10^5 cells/ml for the primary cell strains and 1×10^5 cells/ml for continuous lines. The coverslips were removed on the fourth day after seeding and rinsed once in Dulbecco's B.S.S. Without preliminary drying the coverslips were fixed in cold acetone (Analar grade) for 10 min and then dried in air for 30 min to allow any traces of acetone to evaporate. (Other forms of fixation were also tried in an attempt to develop an indirect immunofluorescence technique for detection of membrane antigens: see Results). Acetone fixed coverslips were stored with dessicant at -20°C until required. Prior to staining, they were re-hydrated in Dulbecco's B.S.S. and a drop of the appropriate serum dilution in 20% beef-brain suspension was applied to each coverslip. They were incubated in a moist box for 1 hour at 37°C and then washed thoroughly in PBS buffer (pH 7.2), with continuous stirring, for 30 min. With care not to let the coverslips dry, a drop of 1/10 sheep anti-rabbit globulin conjugated with fluorescein isothiocyanate was applied, the coverslips were incubated for a further 30 min, washed, and quickly dipped in distilled water. They were then either allowed to dry, or mounted directly in 20% glycerol solution in PBS (pH 7.2) on grease-free microscope slides.

The preparations were examined with a Zeiss fluorescent binocular microscope illuminated with Wotan HBO 200 W super pressure mercury lamp. A 40x (oil) planapochromatic objective was used and the filter system consisted of a UG-1 exciter filter and Zeiss 65 and 44 barrier filters. The degree of immunofluorescence was expressed as $\pm$, +, ++ and +++.

Sections of synovial membrane for immunofluorescence examination were cut in a cryostat at -20°C from snap-frozen material, were fixed in acetone for 10 min and used in the indirect immunofluorescence test as described by Lennette et al. (1967) and Schmidt et al. (1966). The viral antisera were used at 1/40 dilution in 20% beef brain after absorption with human red blood cells and human liver powder. Anti-human IgG, anti-human IgM and anti-human C3 were purchased from Dakopatts A/S Ltd. and used in an indirect immunofluorescence test at dilutions 1/100.

For the examination of synovial and other lymphocytes a different indirect immunofluorescence technique was used (Yoshiki et al., 1974). A volume of 0.02 ml of separated lymphocyte-rich fraction (10^7/ml) was treated with goat anti-sera at a dilution 1:5 for 30 min at 4°C in small agglutination tubes. The lymphocytes were then washed three times in EE medium with no serum. The centrifugation of the agglutination tubes was done with a bench centrifuge specially adapted to take two small plastic racks holding 50 agglutination tubes each. The wash fluid was removed by suction and the washed cells treated

for 30 min at 4°C with 0.02 ml of the optimal dilution of anti-goat IgG FITC. The washing procedure was repeated and 0.02 ml of 20% glycerol in PBS was added to each tube. One drop from each tube was placed onto a grease-free microscope slide and covered with a coverslip (6 mm x 22 mm). The microscope slides were kept in a moist chamber until examined with the Zeiss fluorescent microscope.

(b) Solid phase radioimmunoassay. The technique used was based on that of Forghani et al. (1974) for the detection of Herpes virus antigens. The virus infected control cells or the synovial "fibroblasts" were grown on the bottom of small glass vials (controlled neck shell vials, 9.5/10.5 x 48/50, Johnsen and Jorgensen Ltd., London) in 0.4 ml media. The seeding concentration was 100,000/ml of media. After 4 days' incubation at 37°C the culture contained approximately 50,000 cells per vial. On day 4, after the removal of the growth medium, the cells were rinsed with 1 ml of PBS; they were then fixed in cold acetone for 10 min while still wet. After the removal of acetone the vials were allowed to dry in air to remove any traces of acetone. A volume of 0.1 ml of the antiserum dilution (PBS + 2% FCS) was then added to each vial and incubated for 2 hours at 37°C. The contents of the vials were then aspirated and they were washed four times with PBS. The ^{125}I conjugate was diluted to contain 60,000 counts per 100 seconds (c/100s) in 0.1 ml (PBS + 2% FCS). A volume of 0.1 ml of ^{125}I conjugate

was added to each vial and they were incubated for 80 min at room temperature. The contents were aspirated and the vials were again washed four times. The cell bound radioactivity in the tubes was assayed on a gamma counter (Wallac-LKB counting system).

The solid phase radioimmunoassay for antigen detection described above was adapted for detection of antibody by extending the times of incubation to 16 hours at 4°C for the first antibody reaction and four hours at room temperature for the ^{125}I labelled antibody reaction. The glass vials were seeded with RD-114, RD-114 RD cells, KW23 and KNRK at a concentration of 40,000 cells/vial. The comparison of control positive sera and the human sera from various groups was made after subtracting the counts bound to uninfected cells from the counts bound to the infected cells.

(c) Peroxidase-anti-peroxidase (PAP) method. The synovial tissues for this method were fixed in 4% formol saline and embedded in paraffin. Paraffin sections, 3-4μm routinely processed, (Pathology Department, Edinburgh University) were obtained with care taken to avoid over-heating (temperature never exceeding 56°C).

The peroxidase-anti-peroxidase method used was that of Burns (1975) and is given in the Appendix. The standardisation of the reagents was carried out by Mr B. Hogg (Pathology Department, Edinburgh University) who was using this technique for other

purposes. The modifications needed to detect rubella antigens will be given under Results.

Other serological techniques

(a) Complement-mediated cell cytotoxicity: The test used was that described by Detrick-Hooks et al. (1975). In brief, 0.1 ml of 1.5 x 10^5/ml of lymphocyte suspension was mixed with 0.1 ml of antiserum at appropriate dilution and held at 2 hours at 37°C. The cells were pelleted and 0.1 ml of rabbit normal serum at 1/4-1/6 dilution was added as a source of complement. After two hours at 37°C the dead cells were detected with trypan blue stain. The percentage of cytotoxic killing was determined by placing the cell suspension in a counting chamber in a phase contrast microscope. The percentage of specific virus cytotoxicity =

$$\frac{(\%\text{ of dead cells in rabbit anti-viral serum with complement}) - (\%\text{ of dead cells in absence of complement})}{(\%\text{ of dead cells in rabbit anti-cell serum in presence of complement}) - (\%\text{ of dead cells in absence of complement})}$$

(b) Gel diffusion precipitin test: The fluid from infected cell cultures were concentrated 100 times by dialysis against polyethylene glycol (M 20,000 BDH). The resulting concentrates were tested by immunodiffusion as described by Le Bouvier (1969). Tests were done on 75 x 25 mm glass slides covered with 2 ml of 1% agarose in distilled water. This layer was dried down completely. The coated slides were then covered with 2.4 ml of gel, consisting of 0.45% agarose in 0.1M Tris buffer, pH 8.0, 0.1M NaCl and 15 mM sodium azide. The 3-mm diameter cup held 10 µl of reactant.

Edge-to-edge distance between antigen and antibody cups was 4 mm. Slides were kept in moist boxes at room temperature for 48 hours and if there were no bands within this time, they were discarded. The slides were observed for evidence of precipitin lines at 24 and 48 hours, by viewing against a dark background with a bright light. If precipitin lines were present, the slides were washed in physiological saline for 24 hours, then in distilled water for a further 24 hours and the precipitates stained with 0.1% amido black. Excess stain was removed with a mixture containing acetic acid, methanol and distilled water in the ratio of 10:45:45. The slides were dried down under a filter paper (Whatman, No. 1) soaked in this mixture. The dried slides became a permanent record. However, photographs of the bands were taken from unstained preparations using Ilford film (FP 4) and a dark background.

(c) Immunoelectrophoresis/autoradiography: This technique, a combination of electrophoresis and gel diffusion, is used for separation of human serum proteins. The method employed was Dr W. McBride's modification of Scheidegger's technique (1955). Microscope slides (75 x 25 mm) were coated with 1% agar (Noble, Difco) in distilled water. This layer was dried down on a hot plate and a further 2.5 ml of 1% agar in Veronal buffer (pH 8.2, 0.05M was added. After hardening at 4°C, wells and a trough were cut with a Shandon cutter. Two wells were filled with the synovial membrane and rabbit spleen culture materials from the in vitro radioisotope labelling experiments (see section Organ

cultures), and the microscope slide was subjected to a constant voltage of 250V for 90 min (Shandon electrophoresis apparatus) applied through wicks made from filter paper (Whatman No. 1). To prevent overheating of the agar the supporting plate for the microscope slides was continuously cooled with running water.

At the end of the run the immunoelectrophoretic pattern was developed with anti-human serum (Wellcome, anti-human serum for electrophoresis). The precipitin lines appeared within 24 hours at RT. The slides were washed in saline for 24 hours and in distilled water for a further 24 hours.

Because the culture supernatants (especially from the rabbit spleen) contain too little serum protein to provide a well-defined precipitation line, a carrier serum was added. The antigen well is first filled with the carrier, normal serum. After the serum has been absorbed into the agar (4 min) the culture supernatant was added to the wells and electrophoresis is carried out at room temperature.

The precipitated proteins were stained and dried down as outlined in the gel diffusion precipitin test. After complete dehydration they were brought into contact with sensitive film emulsion (X-ray film, Agfa Gevaert, Osray RP 1). Up to eight microscope slides were placed in an X-ray film holder and the whole sheet of the film and the slides were clamped together. The film was developed after five weeks (D19 Developer, Kodak) and the autoradiographic lines on the film were compared to those

on the immunoelectrophoretic slide. The class of the labelled immunoglobulins was noted and an attempt was made to quantitate the amount of labelled immunoglobulin by marking the intensity of the film emulsion sensitisation with symbols (+++, ++, +, -).

(d) Haemagglutination-inhibition tests. Rubella and measles antibodies: The rubella haemagglutination-inhibition (HAI) tests were done with rubella HA antigen (Wellcome Reagents Ltd) using one day old chick red blood cells as the indicator system. The latter were provided by Dr E. Edmonds (Regional Virus Laboratory, City Hospital) who also kindly performed some of the haemagglutination-inhibition tests. The chick cells, stored in Alsever's solution, were washed three times in dextrose-gelatine-veronel buffer (DGV) and suspended at a concentration of 0.2% and 50% in DGV buffer. To remove non-specific inhibition and agglutinins volumes of 0.1 ml of the test sera were treated with 0.3 ml of manganous chloride/heparin soln. for 20 min at 4°C ($MnCl_2$/Hep. made up by mixing equal volumes of stock solution of 1,000 iu/ml of heparin and 1 M manganous chloride just before use). One drop of a 50% chick cell suspension was added for 1 hour at 4°C. After centrifugation, the supernatant was incubated at 56°C for 30 min and this serum (now diluted 1:4) was stored in -20°C if not used at once for the HAI test. Before the HAI test the rubella HA antigen was titrated in Microtiter "V" plates (Flow Laboratories Ltd) and the antigen was used at 4 haemagglutinating units (HAU) for the sera and eluates and at 8 HAU for the serum fractions. The test proper

was carried out by diluting the sera in the Microtiter plates with microdiluter loops. One drop (0.025ml) of DGV buffer was added to each well, starting with well number 2. The first well contained two drops of the 1:4 dilution of the test sample. One drop of HA antigen was added to each serum dilution, except for the serum control, and the antigen-antibody reaction was allowed to take place for 1 hour at 4°C. One drop of 0.2% day old chick cell suspension was then added and the antibody titres were read when the cells had settled (approx. 3-4 hours). The antibody titre of the serum was taken as that well with the highest serum dilution showing complete inhibition of haemagglutination.

The measles haemagglutination-inhibition test for antibodies was carried out as directed by Wellcome Reagents Ltd. for their measles HA reagents. The indicator red blood cells were from the rhesus monkey. They were washed three times in normal saline each time removing carefully the supernatant containing the white blood cells. When the supernatant was clear, suspensions of 50% and 0.5% were prepared. Eluates from synovial membranes were treated to remove agglutinins for monkey red cells by mixing a volume of each eluate with an equal volume of 50% red blood cell suspension and holding overnight at 4°C. The lyophilised measles HA antigen was re-constituted in distilled water and titrated in microtiter 'V' plates in normal saline with the microtiter dilutors; four HAU were used in the test proper. The latter was set up adding 0.05 ml of the 1:2 dilution to the first well and 0.025 ml of saline to the subsequent wells and

making doubling dilutions. When a synovial eluate was diluted, 4 HAU of measles antigen were added to each well and the plate was allowed to stand for 1 hour at room temperature. After adding 0.5% rhesus monkey erythrocytes the plates were gently tapped to ensure even distribution of the red cells and were incubated for 1 hour at 37°C. The antibody titres were recorded as those dilutions of serum showing complete inhibition of the agglutination. All tests included controls for antigen dose, serum agglutinins and red blood cells stability.

MISCELLANEOUS METHODS

Attempted isolation of rubella virus from synovial specimens

(a) In rabbits. The method was based on that used by Kono (1969) for inoculation of pregnant rabbits with rubella virus. Two small female Dutch rabbits were mated and seven days later each was inoculated with 1 ml of a synovial specimen into an ear vein. The rabbits gave birth on day 22 and the surviving progeny were examined for malformations as a sign of the vertical transmission of rubella infection. The foetal tissues were cultured for virus. The mothers were tested for rubella HAI antibody three weeks after giving birth.

(b) In cell culture. Vero cells and RK_{13} were seeded into roller tubes at a concentration 10^5 cells/tube and rotated at 16 revolutions/hour. After two days the semiconfluent monolayers of Vero cells were washed with EE medium (no serum) and incubated with 0.2 ml of the specimen for one hour at 37°C. The specimen was then removed and replaced by fresh EE medium, supplemented with 2% FCS. The Vero tubes were incubated at 37°C for a further four days and the fluid phase was transferred onto semi-confluent monolayers of RK_{13} cells and absorbed as described previously. The Vero cells were then trypsinised and seeded onto sterile coverslips (6 mm x 22 mm) in tubes. These coverslips were examined by indirect immunofluorescence for the presence of rubella antigens. RK_{13} tubes were examined daily for rubella CPE for nine days.

Separation of materials on sucrose gradients

(a) Virions and immune complexes. Gradients of 70% to 20% sucrose w/v were used. Stock 70% w/v sucrose solution (Analar grade from sugar cane, BDH) was made up in Tris-EDTA buffer (pH 7.2; 0.01M Tris, 1mM EDTA): density 1.27 to 1.28 g/cm^3.

This stock solution was diluted to 60%, 50%, 40%, 30%, 20% with Tris-EDTA buffer just before making the gradients. Two ml of each sucrose concentration was carefully layered in a cellulose nitrate tube (Beckman, Size 9/16" dia. $3\frac{3}{4}$"; vol. 13 ml) starting with the most concentrated solution. Five sharp bands were seen at this stage. The gradients were allowed to equilibrate for 6-24 hours and were discarded if not used within 48 hours. Volumes of 0.2 ml - 1.0 ml of samples with virions were layered on top of the gradient and the tubes were spun at 25,000 rpm for 18 hours (SW 40 rotor, Beckman ultracentrifuge). The run was terminated without braking. The tubes were pierced at the bottom with a needle (25G 5/8) and 6-9 drops were collected into Wassermann tubes to yield 22-26 fractions. A refractive index was determined by measuring one drop from the fractions 6, 11, 16 and 21 on a refractometer (Bellingham & Stanley Ltd., London). The fractions were then precipitated with 1 ml of cold 10% trichloroacetic acid (TCA) ($4^{\circ}C$) and one drop of 2% bovine serum albumin was added to aid the precipitation. Fractions

were placed for 30 min at 4°C and the precipitates were collected on glass fibre filter papers (2.5 cm GF/A, Whatman) using a Millipore sampling manifold attached to an air vacuum pump. The filters were washed twice with 5 ml of cold 10% TCA and once with 5 ml of ethanol, dried to completion (3-4 hours, RT), and placed for counting in vials with 2 ml of toluene based scintillation fluid [Toluene, scintillation grade from BDH was mixed with 0.5% 2.5-diphenyloxazole (PPO) and 0.013% of 1.4-bis-(2-(5-phenyloxazolyl))-benzene (POPOP)]. The vials were counted in a liquid scintillation spectrophotometer (Tri-carb, Packard) for 10 min each. The TCA precipitable counts were then plotted against fraction numbers. The refractive index was converted to sucrose density (g/cm^3) using a standard curve and the sucrose density was plotted against the fraction number on the same graph.

For separation of adenovirus and its immune complexes the sucrose concentration of the bottom layer was increased to 80% and the gradient formed with 1.7 ml of each sucrose concentration (80% - 20%).

(b) Serum proteins. Smaller sucrose gradients were used for separation of serum proteins. 40% sucrose solution was made up in PBS (pH 7.2) and diluted to 30%. 20% and 10% with additional buffer. 1 ml of each sucrose concentration was carefully layered in cellulose nitrate tubes (Beckman, $\frac{1}{2}$"

dia. Size 2") and allowed to equilibrate at 4°C. A volume of 0.5 ml of the serum was placed on the gradient and spun at 35,000 rpm for 18 hours (SW-50.1; Beckman ultracentrifuge). Five drop fractions were collected. The number of fractions varied from 14-20.

For the detection of rubella IgM the serum samples were first diluted 1:2 in dextran glucose veronal buffer (DGV buffer) and two drops of a 50% suspension of washed one day old chick blood cells was added to each serum sample for two hours at 4°C. After centrifugation to remove the red cells (400 g for 10 min), 0.5 ml of diluted serum was inactivated at 56°C for 30 min before layering on the gradient. The collected fractions were used directly for haemagglutination inhibition tests for rubella antibodies.

RESULTS

EXAMINATION OF SYNOVIAL CELLS FOR RUBELLA ANTIGENS

As a preliminary to an attempt to confirm the presence of rubella antigens on synovial cells reported by Patterson, Deinhardt and Howard (1973) it was necessary to establish chronic rubella infection in cell lines so that these could be used to calibrate and control the immunofluorescence and solid phase radioimmunoassay techniques.

For this purpose LLC-MK2 cells and the HPV77 vaccine virus were used. The growth curve of persistent rubella virus infection in LLC-MK_2 cells has been described (Maassab, Veronelli and Hennessy, 1964) and their use for indirect immunofluorescence has been evaluated (Brown et al., 1964).

A semiconfluent monolayer of the LLC-MK_2 (CCL-7: original) cell line in a Roux flask was inoculated with 0.2 mls of HPV-77 vaccine strain diluted to 10 mls with 199 medium without serum. After allowing the virus to absorb for one hour the inoculum was replaced with a fresh medium,which was subsequently changed twice a week. During the first two weeks there was substantial cytolysis and only a few cells from the original monolayer survived. The latter changed morphologically and became fatter. The surviving cells were removed with beads and placed in a Falcon flask (pass number 2). Once established, the cells were split by trypsinisation once a fortnight and, later, once a week.

At pass number 4 the cells were seeded onto coverslips at a concentration of 2 x 10^5/ml and checked for the presence of rubella antigen by indirect immunofluorescence. Approximately 80% of cells showed positive cytoplasmic staining in the form of bright specks of immunofluorescence scattered through the cytoplasm (Plate 1). At pass number 5 the supernatant fluid was titrated on coverslips of RK-13 cells and was infective at a dilution of 10^{-3}.

The presence of rubella virus was also checked by the uridine incorporation method. ^{3}H-uridine (100 μCi) was added to the chronically infected cells for 18 hours. The supernatant fluid was analysed on a sucrose density gradient (SDG) (70%-20%) to check whether the labelled RNA sedimented at a density (1.18 g/cm^3) typical of rubella virions (McCombs and Rawls, 1968). The control uninfected LLC-MK_2 cells were treated in the same manner (Fig. 3). The cells chronically infected with rubella gave several peaks which were not present in controls. One small peak had a density (1.24 g/cm^3) suggesting the presence of mycoplasma. However, both rubella infected and control LLC-MK_2 cells were free of mycoplasma and bacteria on culture. In order to determine which was the 'rubella' peak the cells were grown in the presence of ^{3}H-thymidine (100 μCi) and ^{14}C-uridine (20 μCi) (Fig. 4). On SDG there was a large peak at 1.18 g/cm^3 which labelled with uridine but not thymidine and is likely to represent the rubella virions. The peaks at 1.20-1.22 g/cm^3 contained both incorporated thymidine and uridine,

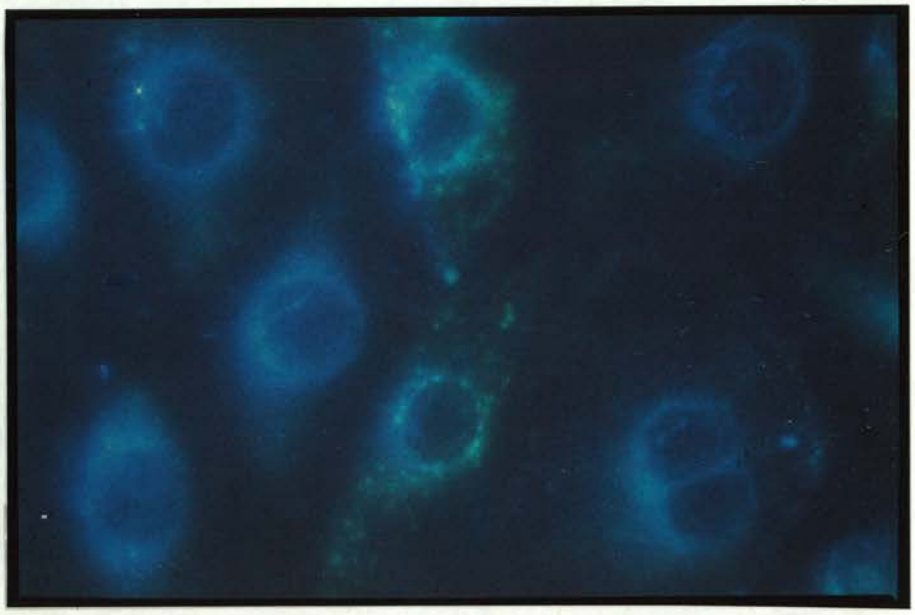


Plate 1: HPV-77-LLC-MK_2 cells, Pass 5; immunofluorescence staining with 1/5 dilution of hyperimmune rubella antiserum R3/HPV-77/6-14.3.75

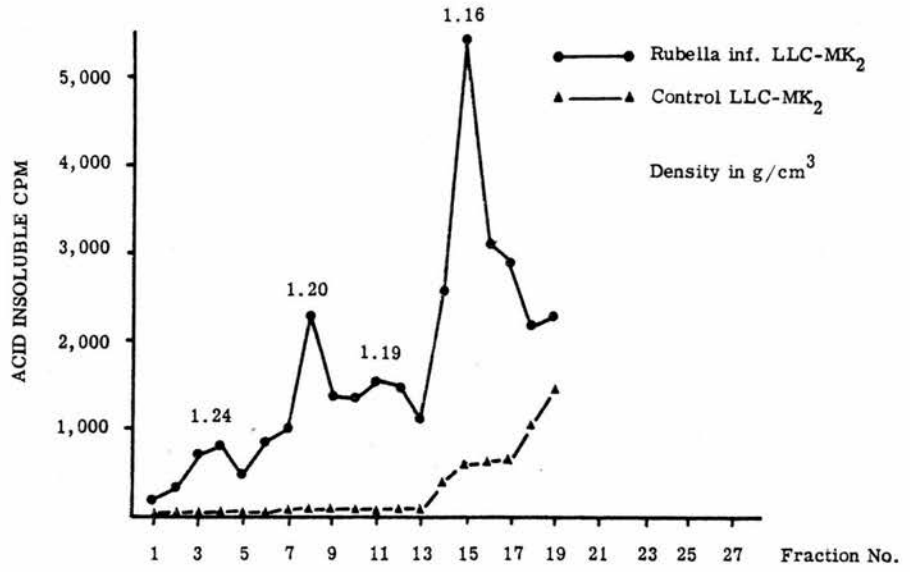


Fig. 3: Acid-insoluble cpm in sucrose density gradient LLC-MK_2 cells chronically infected with rubella (●—●) and uninfected control LLC-MK_2 cells (▲-▲) culture supernatants, labelled with ^{3}H-uridine

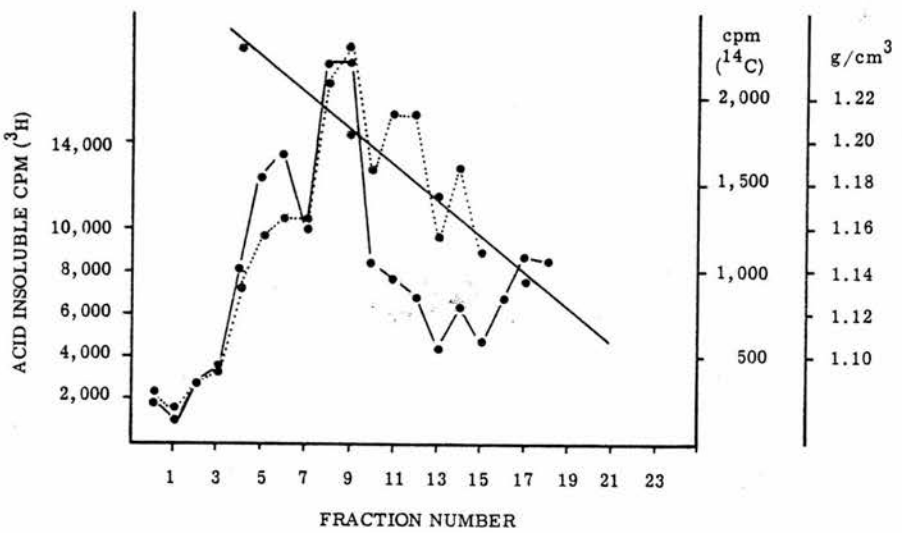


Fig. 4: Acid insoluble cpm in sucrose density gradient LLC-MK_2 cells chronically infected with rubella culture supernatants, labelled with ^{3}H-thymidine (——) and ^{14}C-uridine (-----)

again suggesting the presence of mycoplasma.

From this evidence, it was clear that a culture chronically infected with rubella had been established. This was maintained for one year. During this time the titre of infectious virions in the supernatant decreased and infectivity for RK_{13} cells could be shown only with the neat fluid phase of the LLC MK_2 cells. The number of cells showing positive immunofluorescence also decreased to 3% and simultaneously the cells started to revert to a faster growth rate, similar to that of uninfected cell line.

In later stages of the work a second chronically infected culture was established with the Thomas strain of rubella. This culture showed essentially the same characteristics as the HPV-77-LLC-MK_2 cell line.

Development of indirect immunofluorescence techniques with rubella virus infected cells.

It was decided to examine synovial fibroblasts for internal, cytoplasmic antigen using acetone fixed cells as well as for membrane antigens. Techniques for staining of rubella membrane antigens had therefore to be devised.

Initially, the method of Dr Margaret Haire, Belfast (personal communication) was tried, using unfixed cells grown on coverslips and stained directly with appropriate sera. Detachment of cells during the washing procedure made this technique unsatisfactory. Other methods currently employed for detection of membrane antigens, particularly in measles infected cells, utilise

fixation in 4% formol-saline (Fraser, Shirodaria and Haire, 1974). This technique was tried both with the chronically infected LLC-MK_2 cultures and with BHK-21 cells acutely or lytically infected with rubella virus. The cells were fixed either in acetone, to detect the cytoplasmic rubella antigens, or in 4% formol-saline to detect the membrane antigens. Plate 2 shows the difference in the immunofluorescence patterns by the two methods. On the acetone fixed cells rubella antigens are seen in a form of bright cytoplasmic inclusions outlining the shape of the nuclei while in the formol-saline fixed cells the membrane rubella antigens are seen outlining the shape of the infected cells.

Compared to the previously described acutely infected BHK cells the chronically infected HPV-77-LLC-MK_2 cells showed only weak reactions in membrane staining although cytoplasmic antigen was present. This could be another factor which contributes to the persistence of this virus (Rawls, 1968). The acutely-infected BHK-21 cells and the chronically infected LLC-MK_2 provided good test models for the radioimmunoassay, prior to the main investigation of the synovial fibroblasts.

Development of solid phase radioimmunoassay to detect rubella virus antigens

Solid-phase radioimmunoassay (RIA) will detect viral antigens in infected cells (Hayashi, Rosenthal and Notkins, 1972; Hayashi et al., 1973; Forghani, Schmidt and Lennette, 1974) and

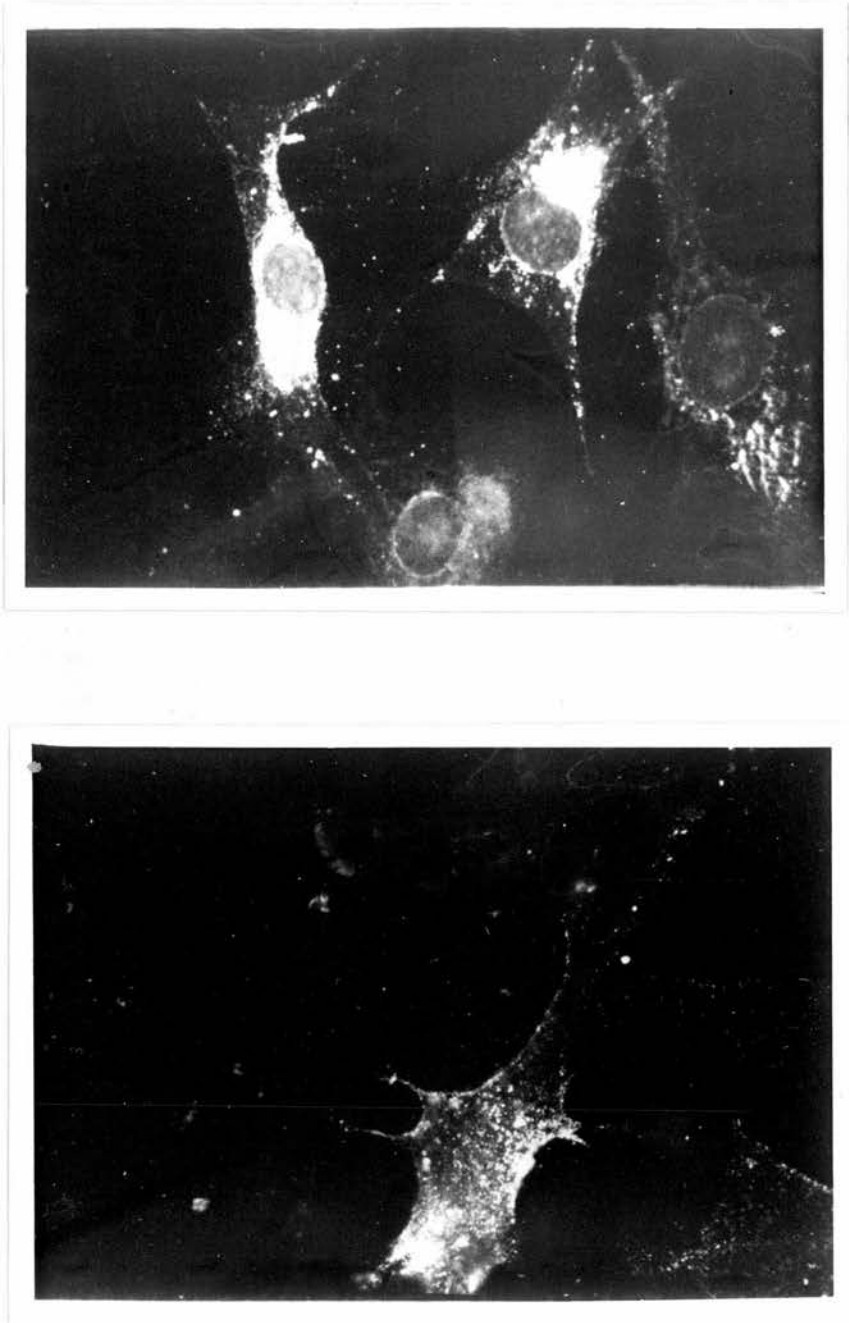


Plate 2: (a) Cytoplasmic accumulations of rubella virus antigen in BHK-21 cells, fixed with acetone and stained with rubella antiserum (R3/HPV-77/6-14.3.75) at 1/5 dilution (b) Membrane antigen of rubella virus in BHK-21, fixed with formol-saline and stained with the same serum

its sensitivity is claimed to be greater than that of immunofluorescence (Hayashi et al., 1973-74). So far it has not been applied to the detection of rubella antigens.

In initial attempts to calibrate the system the rubella (HPV-77) antiserum (R3/HPV-77/6-14.3.75) from the hyperimmunised rabbit R3, and its pre-immunization serum (R3/N2), were titrated by RIA on BHK-21 cells lytically infected with the strain 'Thomas' and also on LLC-MK_2 cells chronically infected with HPV-77. The pre- and post-immunization sera were absorbed with human liver powder, LLC-MK_2 cells and human diploid fibroblasts. The absorbed sera, in half-log dilutions from 1/30 to 1/100,000, were added to cell monolayers on the bottom of glass vials. Goat antirabbit IgG labelled with ^{125}I was used at a dilution giving 60,000 counts per 100 seconds in 100 µl. The resulting titration curves for the two sera on infected and uninfected BHK-21 cells are shown in Fig. 5 and on infected and uninfected LLC-MK_2 in Fig. 6. The number of counts associated with both sets of infected cells that had been treated with antirubella serum R3/HPV-77/6-14.3.75, in dilutions from 1/30 to 1/3,000, was substantially in excess of that on uninfected cells using the same serum. Inspection of the curves shows a titration end point in the region 1/1000 to 1/3000 which approximates to the titre of 1/2048 of the serum as measured by haemagglutination-inhibition before absorption. The control serum (R3/N2) did not bind with either set of infected cells.

An optimal dilution of 1/300 of the antiserum was chosen

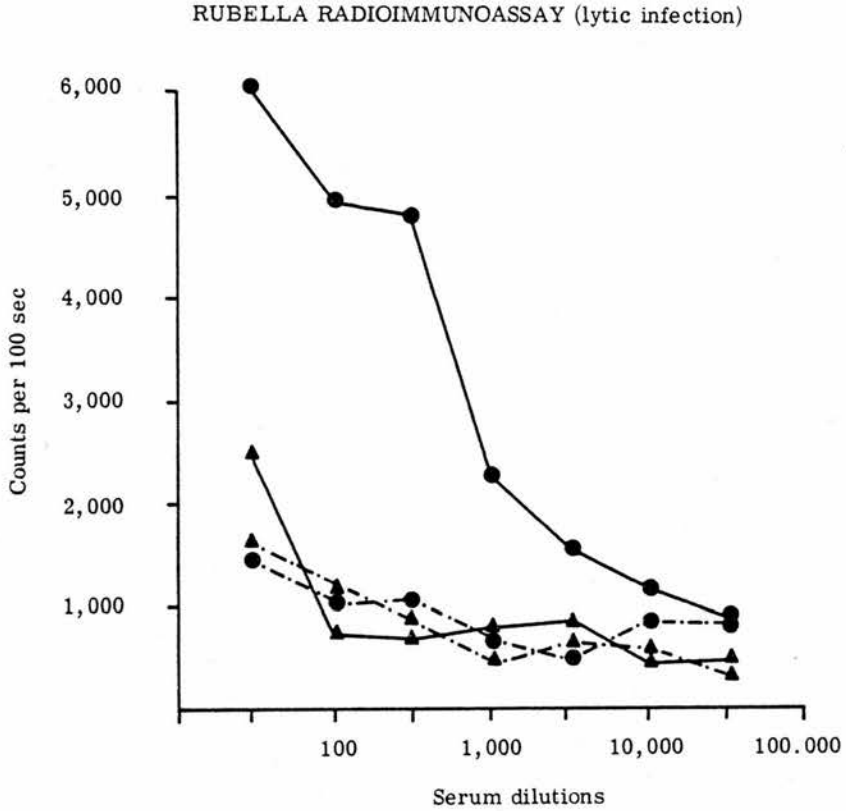


Fig. 5: Titration of rabbit rubella antiserum by RIA on Thomas-rubella infected BHK-21 cells (●-●); control BHK-21 cells (▲-▲); R3/HPV-77/6-14-3.75 hyperimmune rubella anti-serum (—) and R3/N2 pre-immunisation serum (·····).

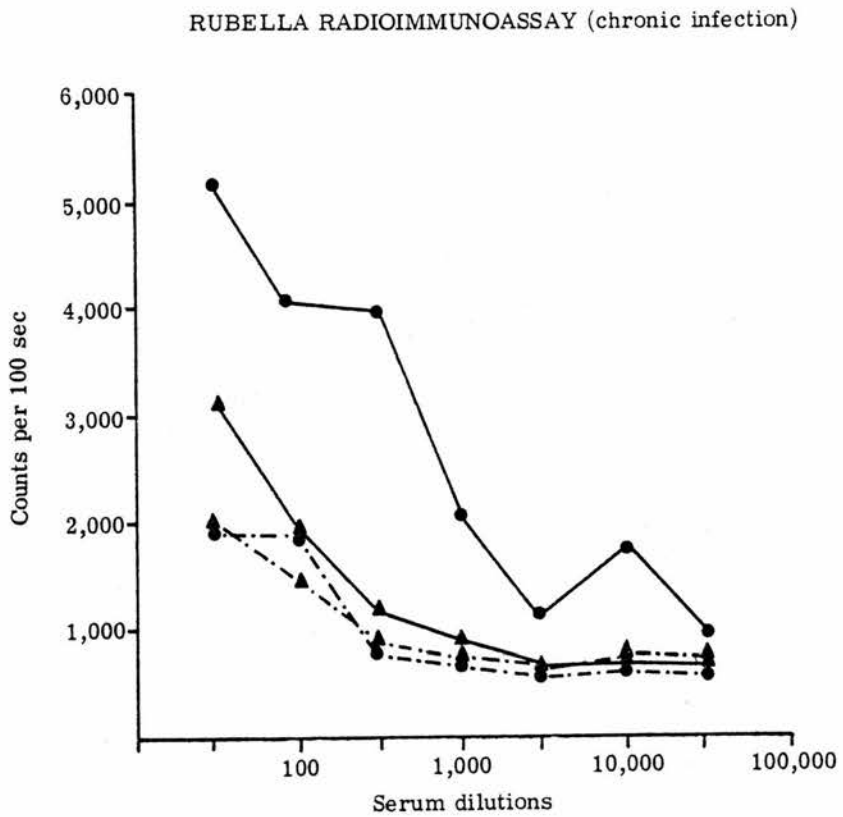


Fig. 6: Titration of rabbit rubella antiserum by RIA on chronically infected LLC-MK_2 cells with HPV-77 rubella (●—●); control LLC-MK_2 cells (▲—▲) of hyper-immune rubella antiserum R3/HPV-77/6-14.3.75 (——) and pre-immunisation serum R3/HPV-77/N2 (-----).

for the test proper, on the basis that the distance separating the antiserum and control curves was greatest at that dilution. The binding antiserum in the BHK-21 cell system can be expressed either (i) as the ratio of counts/100 sec of serum dilution on virus infected cells divided by counts/100 sec on uninfected cells. This gave values of 6.3 for the immune serum and 1.3 for the pre-immune serum. Or (ii) as a ratio, counts/100 sec for rubella antiserum divided by counts/100 sec for the pre-immune serum on the same cells. This gave values of, respectively, 4.5 and 1.0. The chronically infected HPV-77-LLC-MK_2 cells and the controls gave binding ratios of 4.9 and 1.2 by method (ii). These cells had been infected for one year at that time and were in their 22nd passage (about 3.0% of cells showed immunofluorescence at that stage compared with about 80% after the initial infection).

Although satisfactory differences were obtained between the binding of the rubella antiserum (R3/HPV-77/6-14.3.75) on the infected and uninfected BHK-21 cells, these depended on a careful preliminary adsorption of the serum to remove (natural?) heterogenetic antibody against hamster tissue. Figure 7 shows the binding of the serum on infected or control BHK-21 cells before adsorption with acetone dried hamster liver powder and BHK-21 cells. The high 'background' of counts on the uninfected cells contrasts with the values in Figure 3.

The studies of Hayashi et al. (1973) demonstrated the sensitivity of the RIA method for the detection of antibody to

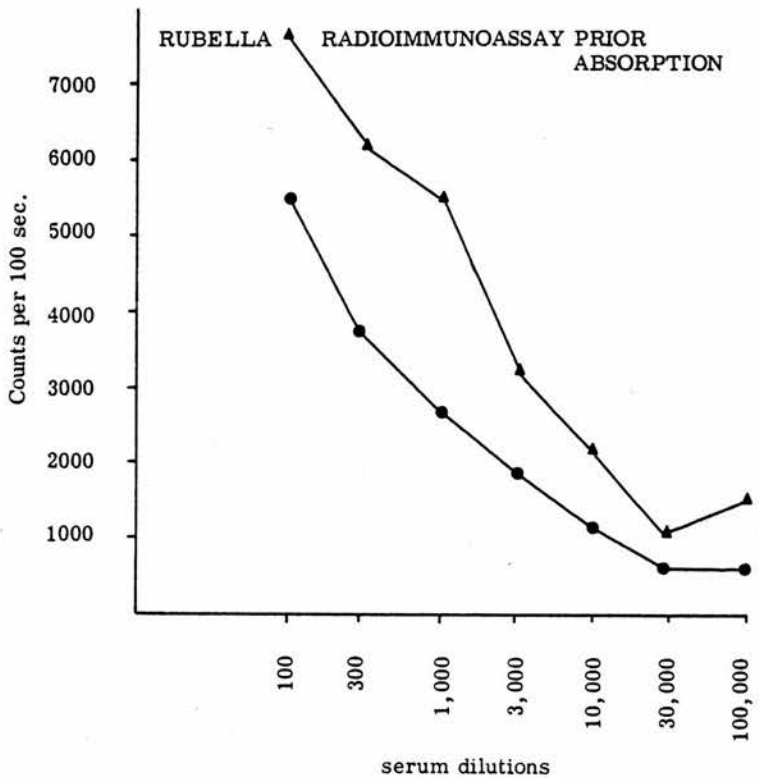


Fig. 7: The binding of the intermediate rubella antiserum R3/HPV-77/6-14.3.75 onto Thomas infected BHK-21 cells (▲-▲) and control BHK-21 cells (●-●) prior absorption of the serum with the uninfected cells.

other viruses. It was important to know its sensitivity, compared to immunofluorescence, for detection of antigen. To investigate this aspect the fluid phase from BHK-21 cells lytically infected with rubella was diluted 10^{0} to 10^{-9}. Unit volumes of the tenfold dilutions were absorbed onto BHK-21 cells grown in vials and onto cells grown on coverslips to be examined by immunofluorescence. Four days after the inoculation all monolayers were fixed and (a) 1/300 dilution of rubella antiserum was layered onto the infected and control BHK-21 cells in vials for RIA, and (b) the coverslips were tested with a 1/10 dilution of the same antiserum by indirect immunofluorescence. Fig. 8 shows the binding ratio (i) plotted against the $\log_{10}$ of rubella virus dilutions. For comparison with immunofluorescence, the intensity of the immunofluorescent staining on the coverslips at each of these dilutions is indicated below with symbols thus: +++, ++, +, -. The end point of the curve is at the viral dilution of 10^{-4} at which point on the corresponding coverslip one bright immunofluorescent cell (IC) was detected. At dilution 10^{-3} which corresponds to approximately 10 rubella infected cells on a coverslip containing usually 10^{5} cells, the binding ratio for the radioimmunoassay test was significantly elevated (2.34). Although it appears from these results that radioimmunoassay and immunofluorescence are of equal sensitivity for detection of viral antigens the former has the advantage of quantitative expression of the results and is more objective.

The sensitivity of radioimmunoassay as measured by the

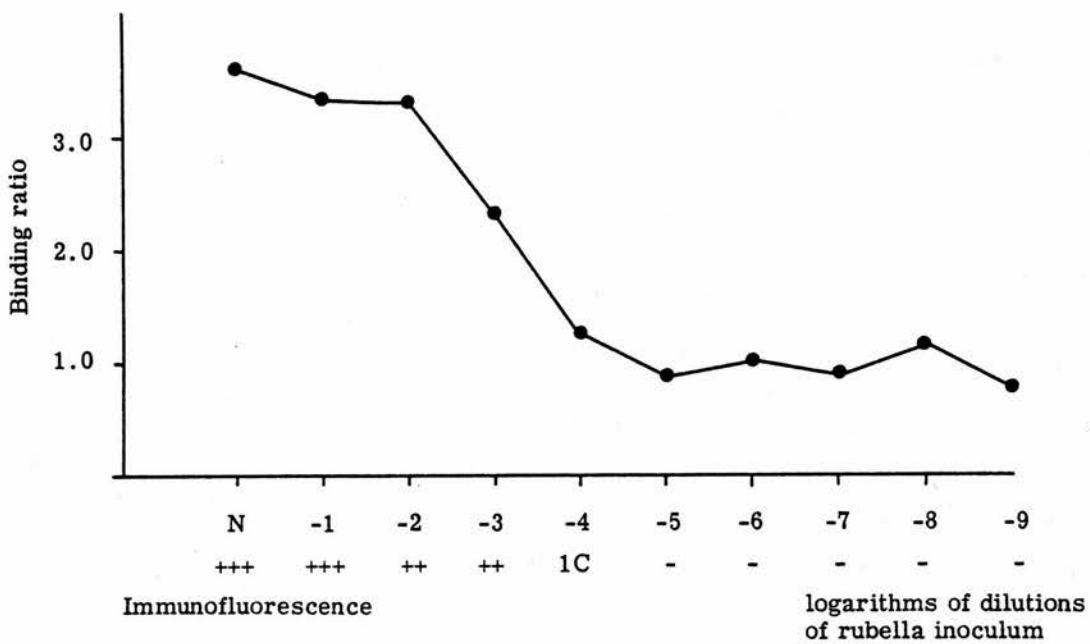


Fig. 8: Binding ratio (i) measured by RIA and plotted against the rubella virus dilutions. The intensity of the immunofluorescent staining is indicated by symbols (+++, ++, +, -) at the appropriate viral dilution.

binding ratio calculated by method (ii) was not increased by the use of larger amounts of goat anti-rabbit IgG labelled with ^{125}I. The larger amounts increased the total counts on the uninfected, control cells as well as on the infected cells (Fig. 9); the binding ratios remained constant. Consequently, 60,000 c/100s of the ^{125}I anti-rabbit conjugate were used in further experiments.

Other investigations of the specificity of the rubella antiserum (R3/HPV-77/14.3.75) revealed some cross reactions with calf serum despite the fact that the RK13 cells used to grow the viral inoculum for the rabbits had been passaged once in rabbit serum before use. It seems from this observation, and from the experience of other workers, that cells may 'carry over' serum from the cell culture medium used in a previous passage, at least in amounts that will stimulate antibody when inoculated into another species. Accordingly, another rubella antiserum was prepared in rabbits, this time using the strain 'Thomas' grown in RK-13 cells that had been taken through four passages by trypsinisation and grown in media containing rabbit serum. The virus in the fluid phase of the cultures was concentrated against polyethyleneglycol and inoculated into New Zealand White rabbits.

Rubella antibody titres (HAI) of 2048 to 4096 were reached and the antisera did not react with calf serum. This antiserum (R29/Thomas/2-23.9.75) was also absorbed with human liver powder and LLC-MK_2 cells; it was titrated on Thomas infected BHK-21 cells and control uninfected cells together with the pre-

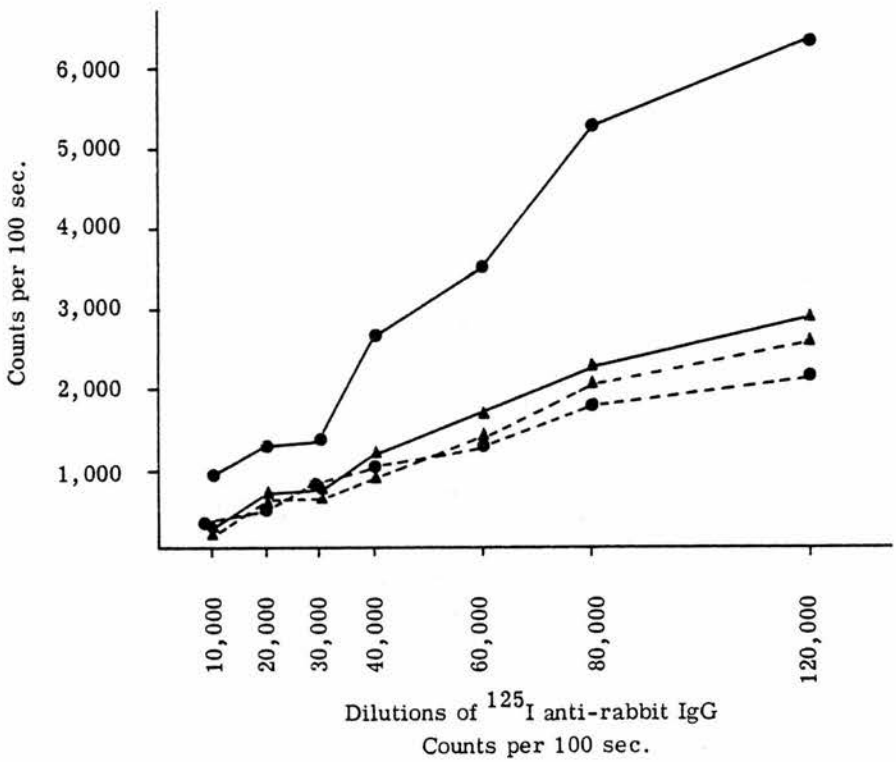


Fig. 9: The effect of ^{125}I-labelled anti-rabbit conjugate on the total counts bound to the infected BHK-21 cells (●-●); control BHK-21 cells (▲-▲) using both hyperimmune rubella antiserum (——) and pre-immunisation serum (·····) at a constant dilution of 1/100.

immunization serum from the same rabbit (R29/N1) (Fig. 10). The titration end point was found again in the region 1/1,000 to 1/3,000; no immunofluorescence was seen with the same serum on uninfected cells or with the pre-immunization serum on the infected cells. This compared favourably with the rubella antiserum (R3/HPV-77/6-14.3.75) previously described.

Attempts to detect rubella virus antigens on synovial fluid fibroblasts

After the standardisation of the rubella antisera prepared in the rabbit, the way was clear to test RA synovial 'fibroblasts' and controls by immunofluorescence and radioimmunoassay.

Immunofluorescence

The rabbit antisera (R3/HPV-77/6-14.3.75) were used at a dilution of 1/20 in testing for cytoplasmic and membrane antigens (see Materials and Methods).

(a) Acetone fixed cells grown on coverslips. The synovial fibroblasts were prepared from eight classical or definite cases of rheumatoid arthritis (RA), seven of osteoarthroses (OA) and one other form of inflammatory joint disease (non-RA). The granular cytoplasmic staining, observed with the chronically rubella infected culture was not detected in the synovial fibroblasts from any source, either with or without hyaluronidase pre-treatment (Table 2).

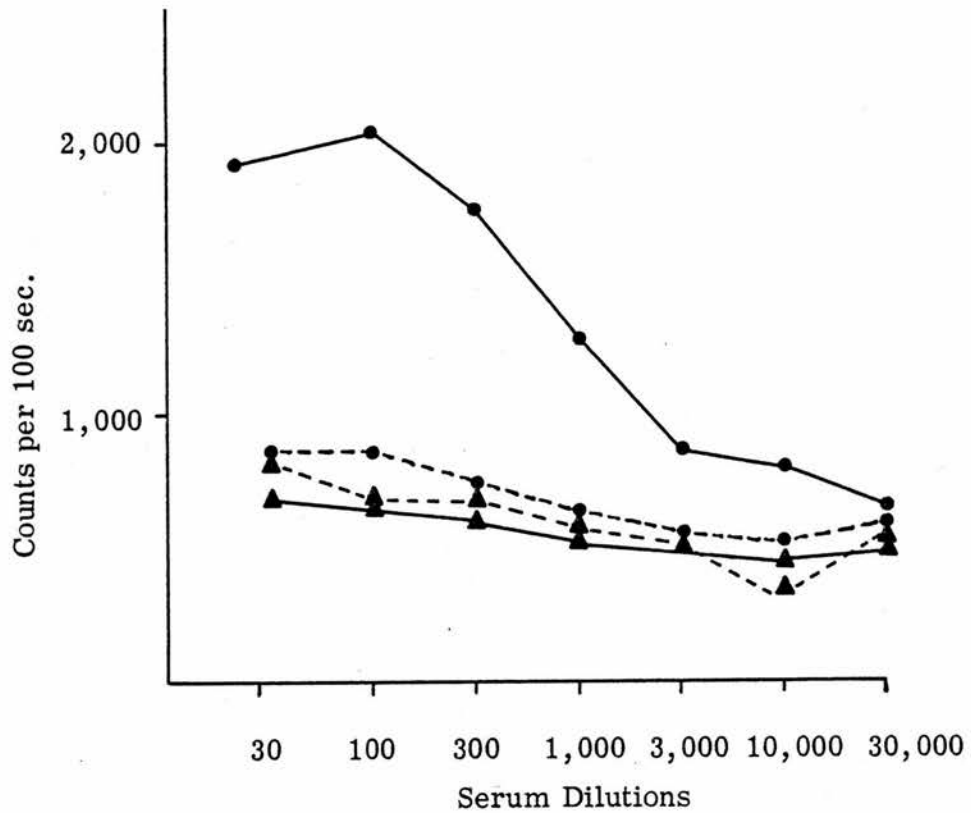


Fig. 10: The titration of rubella antiserum (R29/Thomas /2-23.9.75) on Thomas infected BHK-21 cells (●-●); control BHK-21 cells (▲-▲), R29/Thomas/2-23.9.75) (——); R29/N1 pre-immunisation serum (-----).

Table 2

Culture		Diagnosis	Tissue	Treatment of Fibroblasts	Immunofluorescence Cytoplasmic	Immunofluorescence membrane
A	P10	RA for 3 years	SM	..	-	-
	P10			H	-	-
D	P24	OA	SM	..	-	..
	P24			H	-	..
E	P12	RA for 14 years	SM	H	-	±
F	P14	RA for 14 years	SM	..	-	-
	P14			H	-	±
G	P11	OA for 4 years	SM	..	-	±
	P11			H	-	±
H	P20	RA for 4 years	SM	..	-	-
	P20			H	-	±
I	P15	Polyarthritis (non-RA)	SM	..	-	-
	P15			H	-	-
J	P19	RA for 12 years	SM	..	-	-
	P19			H	-	-
K	P16	OA for 8 years	SM	..	..	-
	P16			H	-	-
M	P10	RA	SM	..	-	..
	P10			H	-	..
N	P13	OA for 3 years	SM	..	-	-
	P13			H	-	-
II	P4	OA	SF*	..	-	..
	P4	4 years		H	-	..
III	P6	RA	SF**	..	-	..
	P6	22 years		H	-	..
IV	P6	RA	SF**	..	-	..
	P6	4½ years		H	-	..
VI	P4	OA	SF**	..	-	..
	P4	50 years		H	-	..
VII	P3	OA	SF*	..	-	..
	P3	8 years		H	-	..
	P3		SF**	..	-	..
	P3			H	-	..
HPV-77-LLC-MK2		P14	Monkey kidney	..	++	-
LLC-MK2		P12		..	-	-
Thomas-BHK-21		P8	Hamster kidney	..	+++	++
BHK-21		P8		..	-	-

TABLE 2: Examination of synovial fibroblasts by immunofluorescence for the cytoplasmic or membrane antigens of rubella virus

Table 2:

K E Y

RA = rheumatoid arthritis

P = passage level of cells

OA = osteoarthrosis

SM = synovial membrane

SF = synovial fluid

SF* = culture was isolated with hydrocortisone (36 g/ml)

SF** = synovial fibroblasts were isolated in the presence of hydrocortisone and sodium aurothiomalate (10 g/ml)

H = hyaluronidase (80 /ml)

.. = not tested or no treatment

- = negative immunofluorescence

(±, +, ++, +++) = increasing degrees of immunofluorescence

(b) Membrane antigens. The technique of 4% formol-saline fixation was used on the collection of synovial fibroblasts from different sources and all gave negative results irrespective of pre-treatment with hyaluronidase (Table 2).

Solid phase radioimmunoassay

Synovial fibroblasts were grown on the bottom of glass vials and exposed to 1/100, 1/300 and 1/1,000 dilutions of antiserum (R3/HPV-77/6-14.3.75) or of the corresponding pre-immunisation serum (R3/N3), and the binding ratios were calculated by method (ii). Fig. 11 shows the binding ratios for 10 RA synovial fibroblasts and 10 non-RA fibroblasts (A to N and II to VII, Table 2), and also the ratios obtained with rubella virus infected BHK-21 cells. There was little difference in the distribution of counts bound between RA synovial fibroblasts on the one hand and non-RA fibroblasts on the other. However, it was known that this particular antiserum had some activity against calf serum (see above). Because of this factor, and also because the collection of synovial fibroblasts was at a high passage level, some fresh isolates (VIII to XXI) of fibroblasts were established and tested with R29/N1 (pre-immunisation serum) and R29/Thomas/2-23.9.75 (hyperimmune) rabbit antiserum which had no activity against FCS. As the antiserum gave similar end points on the titration curve (Fig. 10) compared to R1/HPV-77/4-7.1.75) antiserum it was used at the same three dilutions; see Table 3.

Five samples were used at each serum dilution. Of

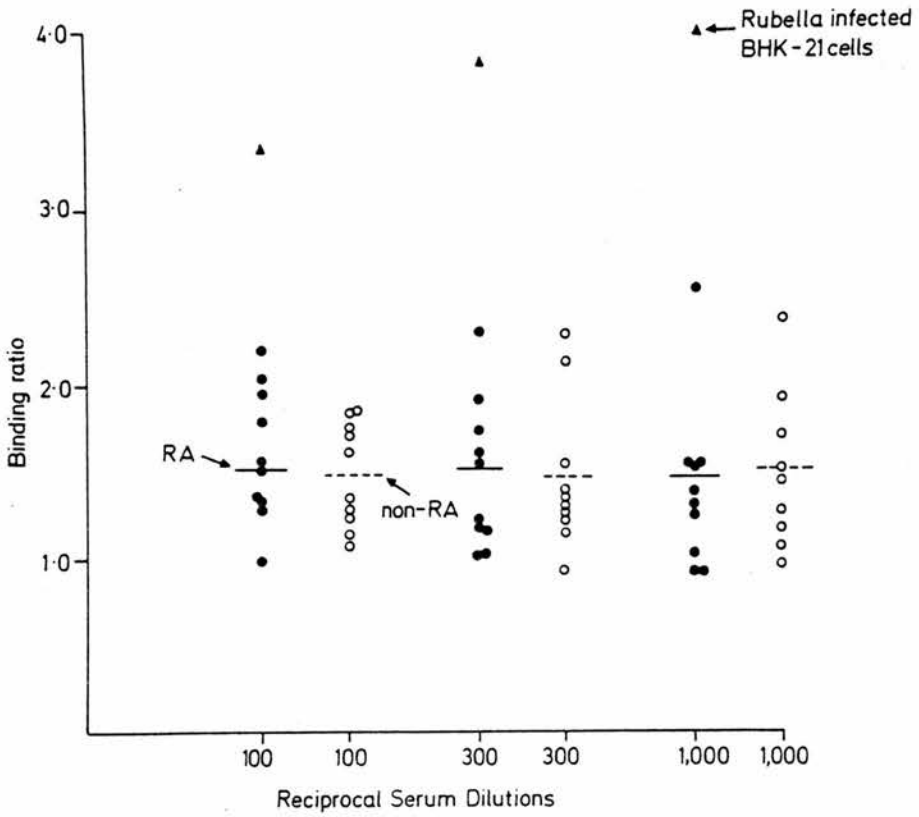


Fig. 11: The binding ratios for each serum dilution (c/100s for hyperimmune serum R3/HPV-77/6-14.3.75 divided by c/100s for the pre-immunisation serum) are plotted in 2 groups:

1. RA fibroblasts(• •)
2. non-RA fibroblasts (o) include osteoarthroses and other forms of inflammatory joint diseases

Table 3

Culture No.	Diagnosis	Serum dilution	Counts bound (± SD) with rubella antiserum (R29/THOMAS/2-23.9.75)	Counts bound (± SD) with Pre-immunisation serum (R29/N1)	Binding ratio
C P17	OA (4 years)	1:100	529 ± 238	415 ± 97	1.27
		1:300	499 + 48	371 ± 73	1.35
		1:1,000	297 + 26	326 ± 43	1
I P19	Poly-arthritis (non-RA)	1:100	597 ± 110	403 ± 36	1.48
		1:300	437 ± 57	408 ± 58	1.07
		1:1,000	317 ± 29	310 ± 8	1.02
N P17	OA (3 years)	1:100	628 ± 84	323 ± 24	1.94
		1:300	381 ± 62	281 ± 22	1.36
		1:1000	309 ± 45	235 ± 32	1.31
V P8	RA ($2\frac{1}{2}$ years)	1:100	699 ± 51	918 ± 54	1
		1:300	512 ± 39	732 ± 73	1
		1:1,000	387 ± 42	525 ± 53	1
VIII P7	OA (8 years)	1:100	436 ± 60	577 ± 63	1
		1:300	336 ± 63	457 ± 46	1
		1:1,000	253 ± 54	335 ± 31	1
IX P6	RA (3 years)	1:100	243 ± 14	229 ± 37	1.06
		1:300	286 ± 23	261 ± 46	1
		1:1,000	180 ± 111	227 ± 37	1

Culture No.	Diagnosis	Serum dilution	Counts bound (± SD) with rubella antiserum (R29/THOMAS/2-23.9.75)	Counts bound (± SD) with Pre-immunisation serum (R29/N1)	Binding ratio
X P7	RA ($6\frac{1}{2}$ years)	1:100	465 ± 64	315 ± 22	1.48
		1:300	315 ± 39	288 ± 36	1.09
		1:1,000	309 ± 172	273 ± 32	1.13
XI P7	non-RA (7 years)	1:100	302 ± 26	407 ± 44	1
		1:300	211 ± 10	260 ± 19	1
		1:1,000	152 ± 29	170 ± 10	1
XII P3	RA (7 years)	1:100	324 ± 50	518 ± 97	1
		1:300	252 ± 31	359 ± 33	1
		1:1,000	218 ± 47	209 ± 46	1.04
XIII P7	RA (6 years)	1:100	450 ± 63	760 ± 67	1
		1:300	316 ± 67	533 ± 181	1
		1:1,000	275 ± 49	340 ± 49	1
XIV P5	prob. RA (6 years)	1:100	527 ± 131	506 ± 28	1.04
		1:300	331 ± 31	367 ± 45	1
		1:1,000	277 ± 41	264 ± 15	1.05
XV P4	RA	1:100	257 ± 43	346 ± 95	1
		1:300	256 ± 71	313 ± 44	1
		1:1,000	219 ± 33	227 ± 60	1

Culture No.	Diagnosis	Serum dilution	Counts bound (± SD) with rubella antiserum (R29/THOMAS/2-23.9.75)	Counts bound (± SD) with Pre-immunisation serum (R29/N1)	Binding ratio
XVI P7	RA	1:100	494 ± 14	505 ± 119	1
		1:300	346 ± 61	321 ± 24	1.08
		1:1,000	252 ± 18	239 ± 31	1.05
XVII P7	RA	1:100	1016 ± 180	1365 ± 196	1
		1:300	829 ± 264	1211 ± 294	1
		1:1,000	818 ± 108	1003 ± 168	1
XVIII P5	RA	1:100	454 ± 80	503 ± 64	1
		1:300	339 ± 24	437 ± 35	1
		1:1,000	249 ± 32	318 ± 58	1
XIX P4	RA (21 years)	1:100	459 ± 37	295 ± 30	1.56
		1:300	374 ± 57	218 ± 20	1.72
		1:1,000	230 ± 19	171 ± 22	1.35
XX P3	RA (10 years)	1:100	505 ± 35	721 ± 43	1
		1:300	361 ± 78	512 ± 29	1
		1:1,000	261 ± 40	398 ± 117	1
XXI P5	Psoriatic arthropathy (4 years)	1:100	526 ± 91	614 ± 39	1
		1:300	429 ± 71	435 ± 46	1
		1:1,000	395 ± 35	423 ± 132	1

Culture No.	Diagnosis	Serum dilution	Counts bound (± SD) with rubella antiserum (R29/THOMAS/2-23.9.75)	Counts bound (± SD) with Pre-immunisation serum (R29/N1)	Binding ratio
CONTROL					
BHK-21 infected with Thomas strain of rubella virus		1:100	1935 ± 141	884 ± 29	2.19
		1:300	1895 ± 129	803 ± 58	2.36
		1:1,000	1313 ± 132	702 ± 110	1.87

TABLE 3: Examination of hyaluronidase-treated synovial fibroblasts from patients with rheumatoid arthritis, and other joint conditions, for rubella antigens detectable by radioimmunoassay with pre- and post-immunisation sera from rabbit 29 given the 'Thomas' strain of rubella virus.

eleven cultures of fibroblasts from patients with definite RA only one culture had a slightly elevated binding ratio (XIX p4). On the other hand, an elevated binding ratio was also found in one culture out of six non-RA fibroblasts (N p17).

In total these results do not support the view that rubella antigens are expressed on synovial fibroblasts from rheumatoid patients.

Rubella antigens on separated synovial fluid lymphocytes

Ficoll-Triosil gradient separated lymphocytes were incubated in medium (E + 10% foetal calf serum) overnight to remove glass adherent cells. Only the supernatant fraction was used in the indirect immunofluorescence test for the detection of rubella membrane antigens on the lymphocytes. The synovial fluid and peripheral blood lymphocytes were treated with hyperimmune rabbit rubella antiserum (R3/HPV-77/6-14.3.75) at 1:10 dilution. The cells were unfixed and the test was done at 4°C to prevent capping of any membrane antigens. There was no membrane immunofluorescence with eight lots of synovial lymphocytes from RA patients and from a case of juvenile rheumatoid arthritis. In two instances the peripheral blood lymphocytes from the same patient were also tested, but with negative results.

Tests for rubella virus antigens in the sections of synovial membrane and cartilage

Synovial membranes from patients with different diagnoses,

fixed in 4% formol-saline were available for an attempt to detect rubella or other viral antigens in the synovial cells. It is known that formol-saline fixed tissues are not very suitable for indirect immunofluorescence, so the technique of immunoperoxidase staining was used. This technique has been successfully applied to detect hepatitis B antigen in liver tissue (Burns, 1975). The first question to be answered was whether the rubella antigens can withstand formalin fixation, followed by embedding in paraffin which may reach a temperature of 56°C for one hour, and still react with the rubella antiserum.

Thomas rubella infected BHK-21 cells grown on coverslips were fixed in 4% formol-saline and then subjected to a temperature of 56°C for one hour. The fixed coverslips were then treated with the rubella antiserum (R29/Thomas/2-23.9.75) followed by swine anti-rabbit IgG and peroxidase anti-peroxidase complex (PAP). Brown colouring (confined to the cytoplasm of the BHK-21 cells) was seen in some of the infected coverslips. The intensity was hardly comparable to that of immunofluorescence but was still recognisable as typical cytoplasmic staining of rubella antigen (Plate 3).

Tissue sections from lung, gut, kidney and liver from a baby with the congenital rubella syndrome were kindly supplied by Dr. I. Smith, Department of Pathology, Royal Hospital for Sick Children, Edinburgh, and were stained by the PAP technique.

The rubella antiserum was absorbed with acetone-dehydrated human liver powder and used at 1/10 dilution in PBS. The liver section showed cells reacting clearly with the rubella antiserum

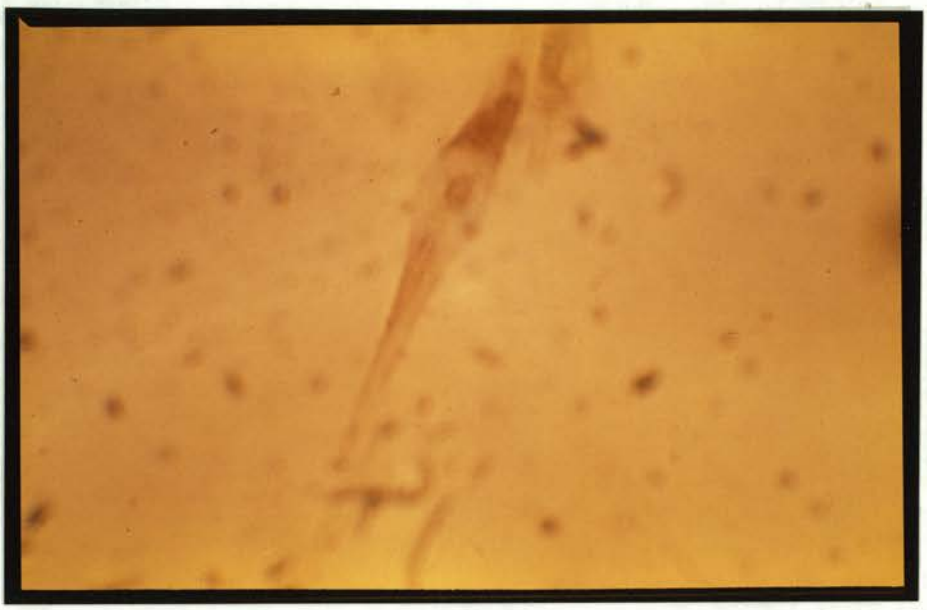


Plate 3: Formol-saline fixed Thomas rubella infected BHK-21 cells stained by peroxidase-anti-peroxidase method. The intermediate rubella antiserum (R29/Thomas/2-23.9.75) was used at 1/10 dilution.

and easily distinguishable once 10% hydrogen peroxide was used to block the non-specific peroxidase activity of numerous erythrocytes (Plate 4). The pre-immunisation serum (R29/N1) did not give the reaction. The reactions became clearer when the incubation period for the antiserum was extended to one hour, in line with the conditions for the indirect immunofluorescence. No cells bearing rubella viral antigens were seen in the other tissues.

This successful application of the PAP technique was extended to sections of three rheumatoid synovial membranes, three synovial membranes from osteoarthroses patients, and three rheumatoid cartilages. There was no brown colouration in any of the cell types present within the proliferations of the rheumatoid synovial tissue or cells found in osteoarthritis (Plate 5). The chondrocytes in the cartilages, as well as the cartilage matrix, were negative (Plate 6).

These results do not support the hypothesis that persistent rubella infection could be the continuous antigenic stimulus in RA.

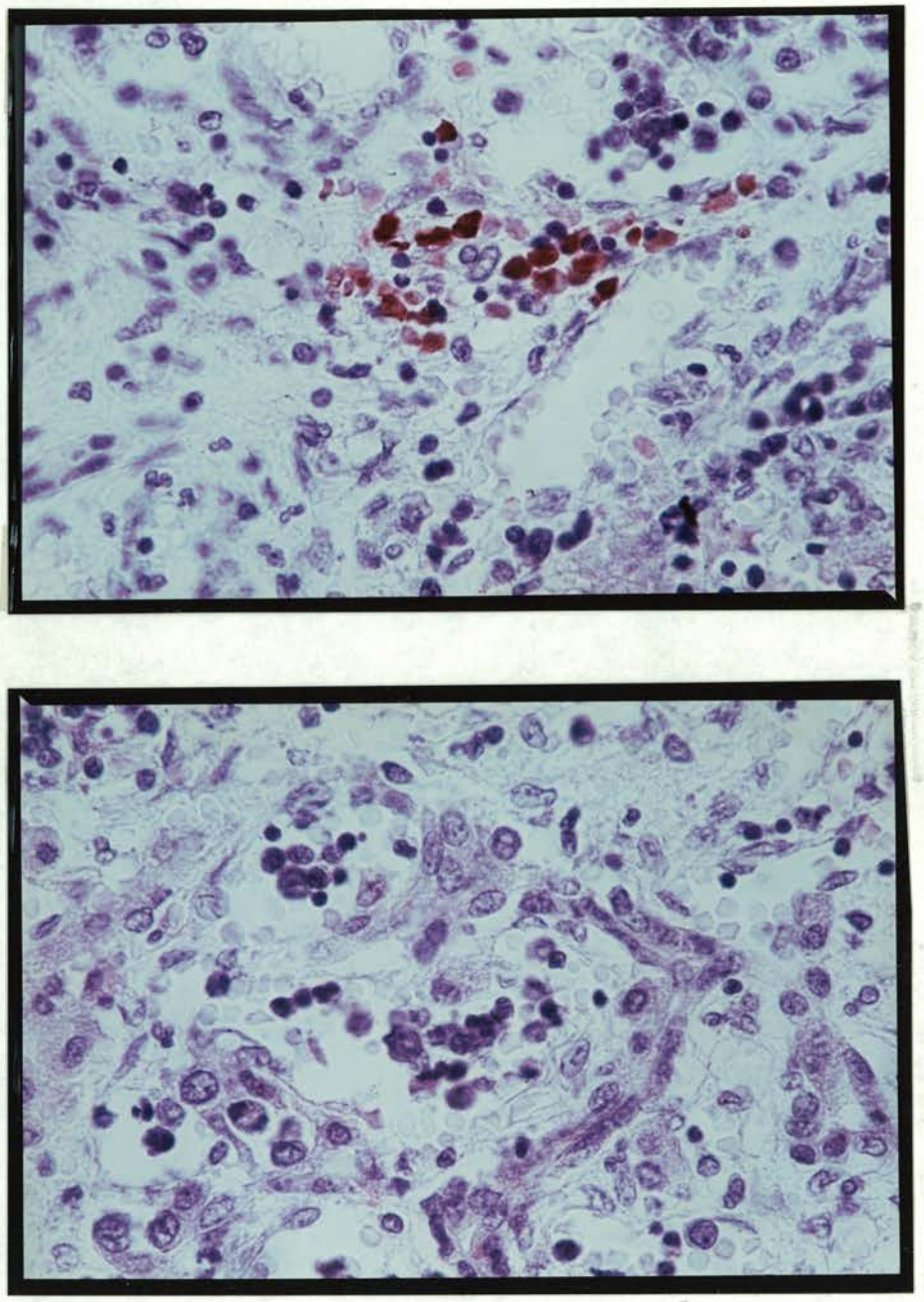
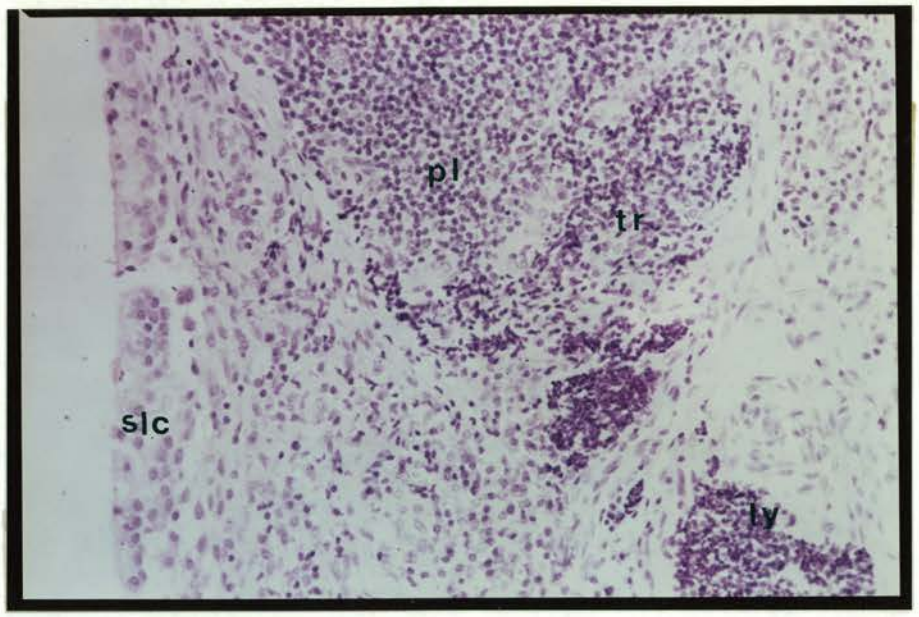
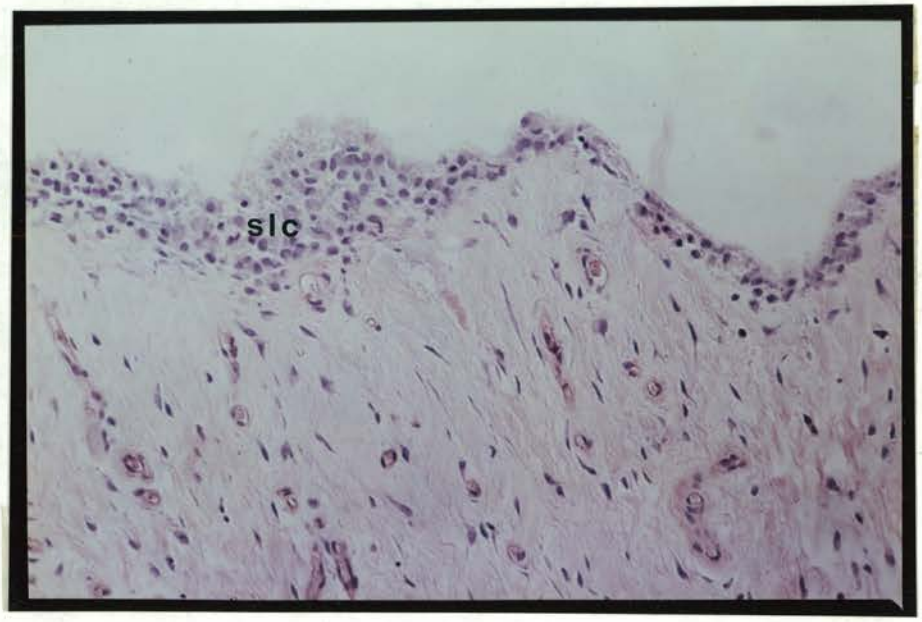


Plate 4: (a) A section of a liver from a baby with congenital rubella syndrome stained by PAP method. The rabbit rubella antiserum (R29/Thomas/2-23.9.75) was used at 1/10 dilution.

(b) The same area of serial liver section stained with the preimmunisation serum (R29/N1).



(a) A section of rheumatoid synovial membrane: pl - plasma cell rich area; ly - small lymphocytes rich area; tr - transitional area with macrophages and lymphoblasts; slc - synovial lining cells.



(b) A section of osteoarthritis membrane: slc - synovial lining cells. No significant proliferation or infiltration with polymorphonuclear or mononuclear cells.

Plate 5: Paraffin sections of synovial membranes stained with rubella antiserum (1/10) by PAP method.

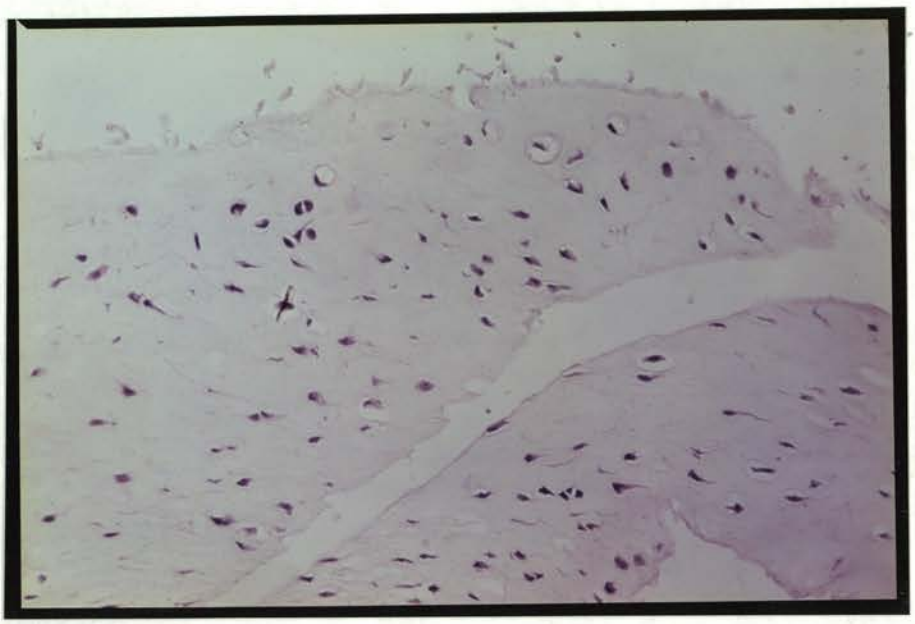


Plate 6: A paraffin section of rheumatoid cartilage stained with 1/10 dilution of rubella antiserum, followed by the reagents of the PAP method.

RHEUMATOID POLYARTHRITIS AFTER RUBELLA

The rubella investigations described previously of the classical or definite rheumatoid adult arthritis gave negative results, but at the time there was considerable interest in the possible involvement of rubella virus in RA stimulated by a particular clinical case that had been described by Dr. J.N. McCormick (McCormick et al., 1978). The case involved a 24-year-old woman who had an illness resembling rubella and subsequently developed an RA-like condition with positive latex and sheep cell agglutination tests and a raised erythrocyte sedimentation rate (ESR). This state lasted for more than 24 months. The virological investigations of the case included immunofluorescence tests for rubella antigens in synovial fluid cells (Dr. McCormick), attempted isolation of rubella from synovial fluid (Hart) and an investigation of rubella IgM in the patient's sera by two techniques: fractionation of sera by acid gel filtration (Dr. McCormick) and separation on sucrose density gradients (Hart). To provide a clearer picture reader is referred to the full paper bound in the back of this thesis.

Tests for rubella antigens in synovial fluid cells (Dr. McCormick)

Specimens of synovial fluid from the knees were obtained on 3 dates (8/9/1971; 4/1/1972 and 28/6/72) were examined for rubella antigens by binding of fluorescein-labelled and radio-labelled

antirubella antibody. A concentrated IgG fraction (rubella HAI titre 512) was prepared by DEAE-cellulose chromatography from a sample of the patient's serum (6/10/1971). Aliquots were labelled with fluorescein isothiocyanate by the dialysis method (Clark and Shepard, 1963) and with ^{125}I by the iodine monochloride method (McFarlane, 1958). Fibroblasts grown in culture from the first two synovial fluids (for 33 and 20 days respectively) and a suspension of washed synovial cells from the third fluid, after the fixation in acetone, were incubated with fluorescein or radio-labelled IgG antirubella antibody but no specific binding was observed (these three samples of cells from synovial fluid, and others taken early in the illness, were subsequently lost by refrigerator failure and were not available for attempted isolation of virus).

Attempted isolation of rubella from synovial fluid

Synovial fluid (23/8/72) for the isolation was taken at the time of persisting rubella IgM in the patient's serum and contained substantial amounts of rubella antibody (see section on IgM antibodies). Two virus isolation methods were used.

(a) In rabbits: The method followed that of Kono et al. (1969) for inoculation of pregnant rabbits with strains of rubella. Two synovial fluid specimens, both collected on August 23, 1972, previously stored in liquid nitrogen, one in Eagle's medium containing 10% D.M.S.O., the other as concentrated synovial fluid cells, were inoculated into the ear veins of two pregnant rabbits

on day 8 after copulation. The rabbits littered 22 days later. None of the 10 neonates showed signs of congenital rubella including eye changes. All neonates died within 2 days of birth; rubella virus was not isolated from their heart, liver, or brain. The rubella HAI titres in the mothers remained negative at 16.

(b) In tissue culture: The synovial cells stored in D.M.S.O. were fused with RK_{13} and Vero cells using UV-inactivated Sendai virus (Watkins, 1971) in efforts to circumvent the possible neutralising effect of rubella antibody in the fluid. Fused and control cells were split once a fortnight and observed for 2 months. At fortnightly intervals fused Vero cells were tested by immunofluorescence with rubella antiserum and antirabbit Ig-FITC as described previously. No rubella antigens were seen in Vero cells and no cytopathic effect was detected in RK_{13} cells.

Rubella antibody in the patient's serum and synovial fluid

Sera. 19 samples of serum collected over a period of 68 months were available. 10 of these samples, obtained between May 1971 and February 1972 were fractioned under acidic conditions by gel filtration on Sephadex G200 columns.

Fractionation of sera by gel filtration. To overcome a significant contamination of IgM fractions with IgG, obtained during fractionation at pH 8, presumably because of complex formation between IgM rheumatoid factor and IgG, all fractionations were carried out at pH 4.0 in glycine/HCl buffer. The individual fractions from the

ascending limb of the excluded first peak were pooled, the pH was adjusted to 7.0 with 0.1M NaOH, and the pool concentrated by ultrafiltration. Bovine serum albumin was added to a final concentration of 1.0% to reduce denaturation of IgM. These fractions were shown to be free of IgG by immunodiffusion analysis. To check that separation of IgM from IgG was complete, IgG prepared from the patient's serum by fractionation on DEAE-cellulose (DE 52) was labelled with ^{125}I by the iodine monochloride technique, and added to the serum before fractionation. On the 4 occasions when this additional safeguard was employed (sera 3, 4, 6, & 11), the fractions in the ascending limb of the first peak were shown to be free of IgG by absence of radioactivity as well as by immunodiffusion. The results are given in the Table 4 ; rubella IgM activity was found in the majority of the IgM fractions prepared in this way. In addition, rheumatoid factor was detected by the tube latex test at a titre of 1/5120 or greater in the IgM fraction of the 4 sera tested (1, 4, 7, & 10).

The results of less satisfactory separations of IgM and IgG rubella antibody on sucrose gradients, utilising sera taken later in 1972 and 1973, indicated a slow fall in IgM levels over this period (Table 4).

Synovial fluid. Only one specimen of synovial fluid, obtained on August 23, 1972, was tested for rubella HAI antibody and had an antibody titre of 512. By an indirect immunofluorescence system using BHK_{21} cells infected with rubella virus and an anti-IgM

Table 4

Serum no.	Date	Whole serum				IgM fractions separated on Sephadex G200 at pH 4.0		IgM fractions prepared on sucrose gradients: rubella HAI titre
		Rubella		SSCT	Latex	Rubella HAI titre	Tube latex titre	
		HAI	CF					
1	13/ 5/71	512	32	512	5120	2	5120	4
2	26/ 6/71	2048	..	512	5120	-	-	-
3	14/ 7/71	2048	..	256	5120	128*	-	-
4	22/ 7/71	2048	32	..	..	64*	5120	-
5	8/ 9/71	1024	..	128	..	2	-	-
6	6/10/71	2048	64	..	..	32*	-	8
7	3/11/71	2048	..	1024	5120	4	5120	-
8	17/12/71	1024	64	..	..	64	-	-
9	4/1/ 72	2048	..	64	5120	64	-	-
10	24/ 1/72	..	..	-	..	64	5120	-
11	23/ 2/72	..	..	32	..	64*	-	-
12	26/ 4/72	1024	..	16	20	-	-	8
13	23/ 8/72	1024	..	..	..	-	-	4
14	7/ 3/73	64	..	64	..	-	-	2
15	6/ 6/73	16	..	256	..	-	-	2
16	15/12/73	256	32	32	..	-	-	-
17	3/ 7/74	..	..	16	20	-	-	2
18	15/ 1/75	..	..	16	20	-	-	-
19	19/ 1/77	128	32	16	20	-	-	-

- = not done; * ^{125}I-IgG added before fractionation but not detected in IgM fraction. .. = not tested.

TABLE 4: Rubella HAI titres on whole serum and IgM fractions prepared on Sephadex G200 at pH 4.0 and on sucrose gradients.

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conjugate, rubella IgM antibody appeared to be present in this synovial fluid. However, it is known that rheumatoid factor will react with specifically bound IgG antibody in immunofluorescence systems (McCormick, 1962) and it has also been suggested that the presence of rheumatoid factor will interfere with the detection of IgM antibody to virus antigens (Shirodaria et al., 1972).

EXAMINATION OF IMMUNOGLOBULINS SYNTHESIZED BY RHEUMATOID SYNOVIAL MEMBRANES FOR VIRAL ANTIBODY

If the pathological changes in rheumatoid arthritis represent an intense immunological reaction against structural cells of a joint altered by virus-coded or other neoantigens, then it should be possible to detect the local production of specific antibody against these antigens. Such antibody might, for example, be found in acid or high salt eluates from washed RA synovial membranes, in cryoprecipitates formed from antigen-antibody complexes in the joints, in the fluid phase of in vitro "organ cultures", or in surviving fragment cultures of synovial membrane of the Maitland-type.

The experiments in this section of the thesis are concerned with the development of sensitive serological methods for the detection of viral antibody in such materials, first with a model system of tissue from rubella virus infected rabbits and, later, with materials synthesised by, or eluted from, RA synovial membranes.

The *in vitro* production of ^{14}C-labelled rubella antibody

Two New Zealand rabbits were immunised with PEG concentrated rubella inoculum (as described under Materials and Methods for the production of specific rubella antiserum). The first rabbit was given two injections and the second animal three injections at weekly intervals. Two days after the last inoculations, the spleen and cervical lymph nodes were removed, dissected free of fat and extraneous tissue, cut into small fragments, and incubated *in vitro* in Eagle's medium containing rabbit serum and a variety of ^{14}C-labelled amino acids overnight. The freeze dried supernatants of these cultures, after reconstitution in distilled water, were tested for the presence of ^{14}C-labelled immunoglobulins by a combination of immunoelectrophoresis and autoradiography; both spleen lymph nodes supernatants were found to be positive. The supernatant fluids of the cultures from rabbit (2) were chosen for further studies because a rise in HAI rubella antibody titre was demonstrated in this rabbit's serum ($<$ 16 to 128) while the first rabbit remained negative.

The following techniques were used to detect locally produced radiolabelled antibody:

(a) radioimmunodiffusion, (b) autoradiography, and (c) sucrose gradient analysis of immune complexes containing ^{3}H-labelled viruses and ^{14}C-labelled immunoglobulins.

(a) *Radioimmunodiffusion* has been successfully applied to analyse the specificity of Ig's synthesised *de novo*. An antigen is precipitated with a known specific antibody to form a

precipitating line in the gel. The unknown ^{14}C-labelled antibody is then allowed to migrate through the gel. The specific reaction of the labelled immunoglobulin with the precipitated antigen is detected by autoradiography. This seemed to be a method with potential, especially since rubella is claimed to have at least two precipitating antigens (Le Bouvier, 1969). Accordingly, PEG-concentrated supernatant from chronically infected rubella cell cultures (LLC-MK_2 cells), which was known to have an infectivity titre of 10^2/ml, was allowed to diffuse against a specific rabbit rubella antiserum R1/HPV-77/4-7.1.75 (HAI titre 4096). There were three bands which all gave a line of identity with foetal calf serum and were clearly non-specific. No additional lines were seen. The reasons could have been (i) not enough antigen, and (ii) absence of precipitating antibody in the rabbit hyperimmune rubella antiserum. An attempt was then made to grow rubella virus to high titre in Vero cells, both in foetal calf serum (FCS) and horse serum (HoS); the latter to avoid the cross-reaction with calf serum. Vero cells in roller cultures are claimed to be the best substrates for growth of rubella virus to high titre (Liebhaber, Pajot and Riordan, 1969). Vero cells, free of mycoplasma, were inoculated with the HPV-77-LLC-MK_2 supernatant. The fluid phase from the Vero cells (HPV-77 in FCS, control cells in FCS, HPV-77 in HoS, and control cells in HoS) were harvested daily and assayed on RK_{13} coverslips for rubella cytoplasmic effect (CPE). Fig. 12 shows the rubella virus infectivity titres in the various Vero cultures plotted against the number of days after the initial inoculation. The highest levels of virus were reached

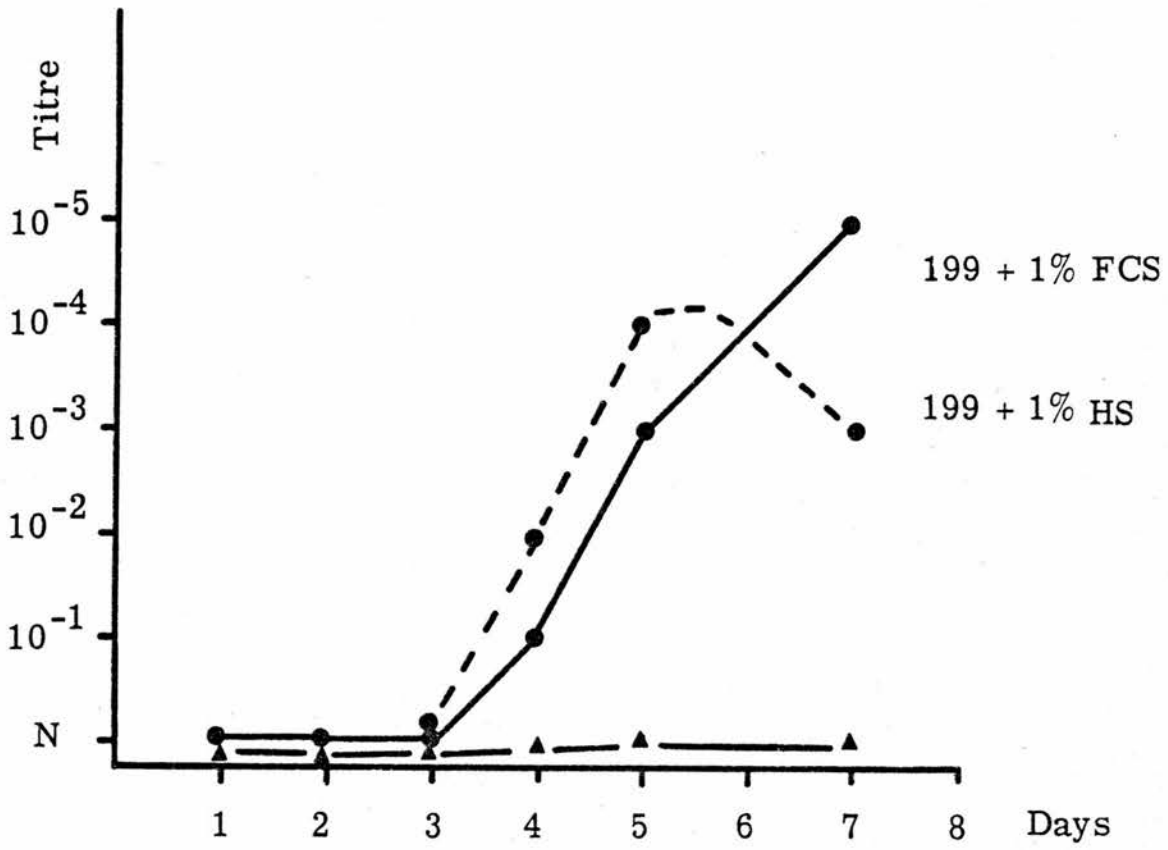


Fig. 12: The growth to high titres of rubella virus in Vero cells. The supernatants of rubella infected Vero cells (•-•) grown in FCS (——) and horse serum (-----) were titrated on coverslips of RK_{13} cells. Uninfected Vero cells (▲-▲) supernatants were negative.

between days 5 to 7. The fluid phases from the infected Vero cells with the highest rubella titres 10^4 - 10^5 were concentrated with PEG and tested by gel immunodiffusion with rabbit antisera. No bands were found with the rubella grown in horse serum, while with the rubella grown in foetal calf serum only the cross-reacting lines were detected. This was very surprising as the virus antigen concentrations used were in the range indicated by Le Bouvier (1969) and the failure raises doubts about the specificity for virus of the results reported. No lines were obtained with another rabbit anti-rubella serum, R29/Thomas/2-23.9.75, known to be free of cross-reactions with foetal calf serum. The absence of the precipitation might conceivably have been due to the lack of a precipitating antibody in the rabbit sera so various human rubella convalescent-phase sera were tried, including a pool of 15 human sera (kindly donated by Dr. E. Edmond, City Hospital, Edinburgh) but without success.

As the formation of a rubella specific precipitin band was essential for this technique to be used as a test of locally synthesised immunoglobulins, it was abandoned.

(b) Autoradiography: The method is similar in principle to that of direct immunofluorescence, except that the conjugate is labelled with ^{14}C rather than fluorescein. Acetone fixed coverslips of rubella-infected cells (HPV-77-LLC-MK_2 p 13; and LLC-MK_2 p 9) were incubated with doubling dilutions of ^{14}C-labelled supernatants from the rabbit spleen lymph node cultures in PBS + 2% FCS for 1 hour at 37°C, washed, and mounted with cells uppermost. They were

then coated with autoradiographic emulsion. The stripping film emulsion was developed after six weeks of exposure but no black granular deposits were found in the cytoplasm of the infected cells.

(c) Sucrose gradient analysis of double labelled immune complexes: 'Thomas' rubella virus was grown in Vero cells for three days (one, 2.5 l roller bottle) and was labelled with 0.2mCi of ^{3}H-uridine for 24 hours. The fluid phase of the culture was concentrated by ammonium sulphate precipitation and spun on a sucrose gradient (70% - 20% w/v). Labelled fractions at the density 1.18g/cm^{3} corresponding to rubella virions and absent in the fluids from control uninfected Vero cells, were pooled from four gradients and stored at -70^{o}C until required. When required for an experiment these sucrose fractions of the rubella virus were diluted (in Tris-EDTA buffer) and pelleted at 35,000 rpm in SW50 rotor (Beckman Ultracentrifuge). The pellets were resuspended in PBS pH 7.2) to a final volume of 0.4 ml; 5 µl of this ^{3}H-uridine labelled material, placed on a paper disc precipitated with 10% TCA and washed with ethanol, had an activity of 370 cpm.

10 µl of ^{14}C supernatant from the rubella primed rabbit spleen lymphnode preparations had a TCA precipitable count of 1470 cpm.

(i) 50 µl of ^{3}H-uridine rubella virions (~ 3,000 cpm) and 50 µl of ^{14}C-rabbit immunoglobulin (~ 7,000 cpm) were mixed. Controls included (ii) 50 µl of rubella + 50 µl of sucrose gradient buffer; (iii) 50 µl of ^{14}C rabbit spleen-lymphnode immunoglobulins + 50 µl of buffer and, (iv) 50 µl of ^{3}H-rubella virions + 50 µl of unlabelled hyperimmune rabbit rubella antiserum (1/10 dilution R27/Thomas/

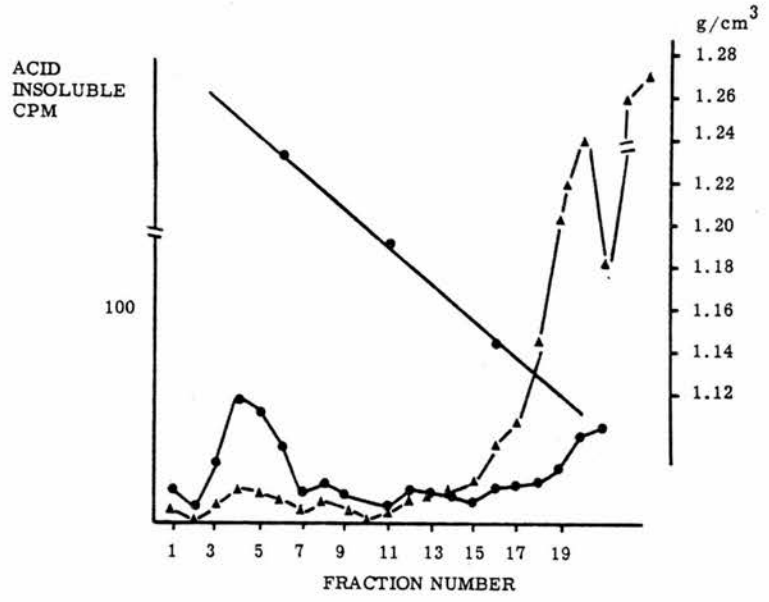
2-23.9.75).

To aid the formation of immune complexes 20 µl of fresh guinea pig serum was added to each of these mixtures to provide a source of complement. All mixtures were incubated at 37°C for one hour and then overnight at 4°C. The resulting reaction mixtures were layered on four sucrose gradients and the TCA precipitable counts in each fraction determined after centrifugation. Each sample was counted for ^{3}H and ^{14}C counts. The ^{3}H-rubella virions retained the density of 1.18 - 1.19g/cm^{3} (Fig. 13(ii)), unless they were mixed either with the rabbit spleen-lymphnode immunoglobulin (Fig. 13(i)) or the rubella R27/Thomas antiserum (Fig. 13(iv)). Then they shifted to a new density of 1.25 - 1.26g/cm^{3} and there was no detectable radioactivity left at 1.18g/cm^{3}. The heavier peak also contained ^{14}C counts which were absent from the same density when the spleen supernatant was spun on its own (Fig. 13(iii)).

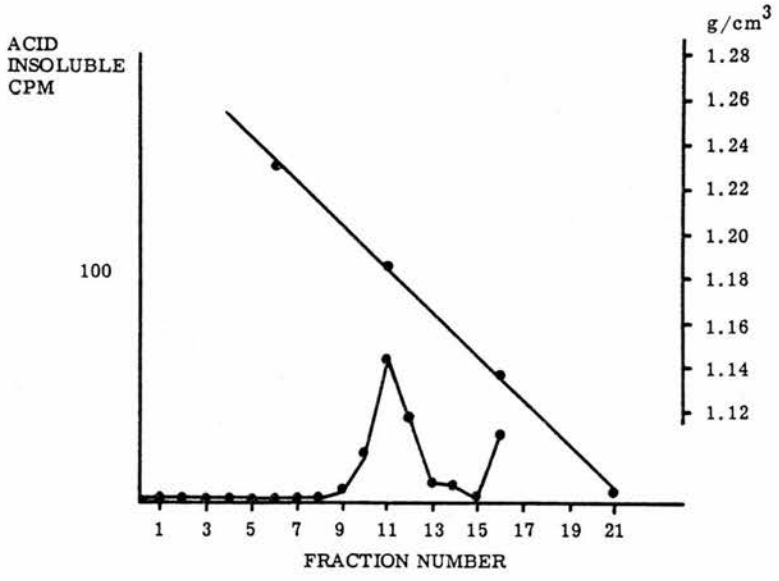
The formation by the rubella virions of a complex sedimenting at a differing density provides evidence that a specific reaction is taking place and the presence of ^{14}C counts in this complex indicates that the complex contains specific rubella antibodies produced <u>in vitro</u> by the rabbit spleen-lymphnode preparation.

To check the specificity of the reaction for rubella, ^{3}H labelled measles virus was prepared and analysed in the same manner (Fig. 14). Fractions over the range of density 1.18 - 1.22g/cm^{3} were pooled from two gradients and concentrated by pelleting at 35,000 rpm and reconstituted to a volume of 0.8 mls.

Volumes of 100 µl of labelled measles virions (2,500 cpm)



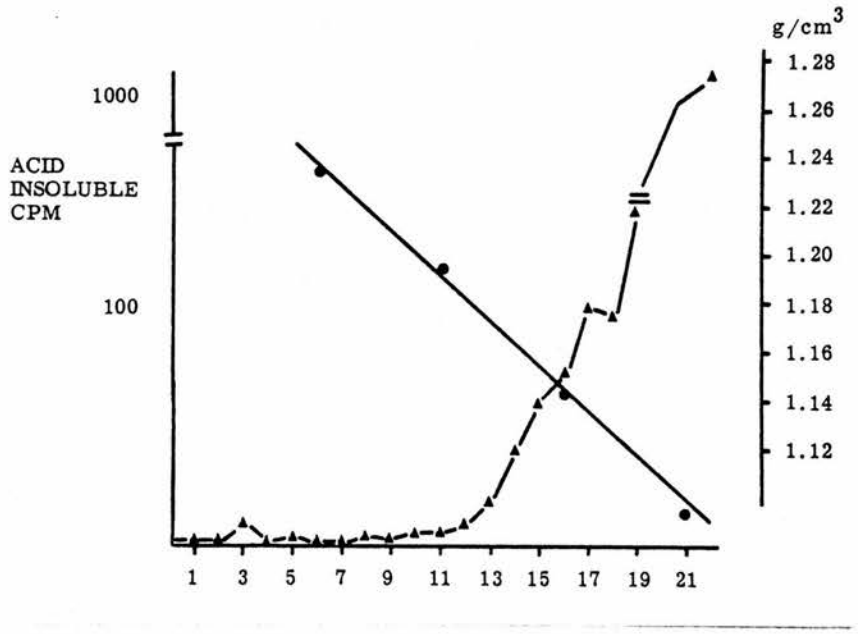
(i)



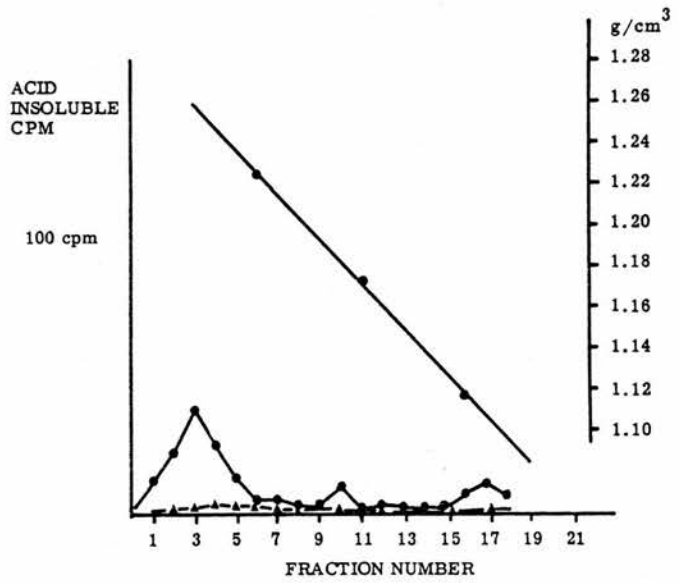
(ii)

Fig. 13: Acid-insoluble cpm in sucrose density gradient fractions of (i) ^{3}H-uridine labelled rubella (•—•) complexed with ^{14}C-labelled spleen/lymph node supernatant from a rubella primed rabbit (▲—▲).

(ii) ^{3}H-uridine labelled rubella control (•—•).



(iii)



(iv)

Fig. 13: Acid-insoluble cpm in sucrose density gradient fractions of (iii) ^{14}C-labelled supernatant from a rubella primed rabbit spleen/lymphnodes.

(iv) ^{3}H-uridine labelled rubella complexed with rubella antiserum R27/Thomas/2-23.9.75.

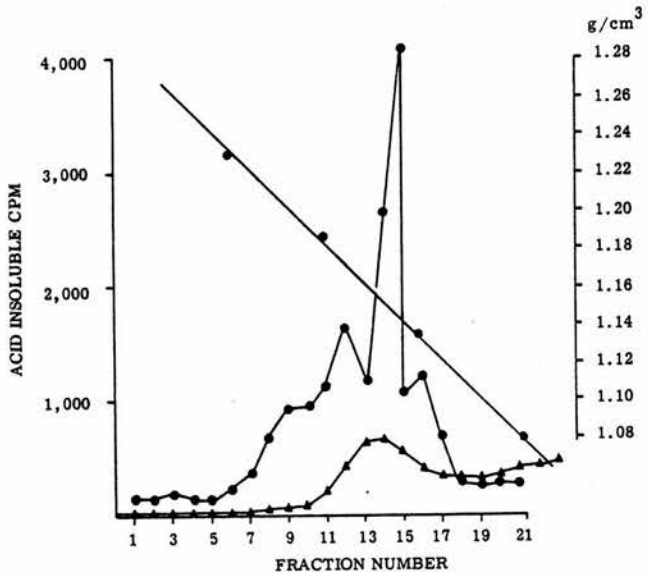


Fig. 14: Measles infected Vero cells (●—●) and control Vero cells (▲-▲) were labelled with 0.1 mCi ^{3}H-uridine. 0.4 mls of the ammonium sulphate precipitated supernatant was spun on a sucrose gradient (70%-20%).

and 20 µl of complement were mixed with (i) 50 µl of 1/10 dilution of anti-measles IgG, or (ii) 50 µl of buffer, or (iii) 50 µl of the same ^{14}C-labelled SN as was used above (7,000 cpm). From Fig. 15 can be seen that new peaks were formed in mixture (i) 1.26 and 1.22g/cm^3 with a shoulder at the density of 1.19g/cm^3, indicating a formation of a measles virus-antibody immune complex, while in (ii) and (iii) only peaks of 1.20g/cm^3 density could be detected. In addition in (iii) ^{14}C counts were not detectable in this peak, confirming that the reaction in Fig. 13(i) was specific for rubella.

It therefore seemed that the double labelling immune complex detection would be a useful technique for the analysis of in vitro produced immunoglobulins from rheumatoid synovial membrane fragments held in short-term culture.

Examination of proteins synthesized in vitro by RA synovial membrane fragments for specific virus antibody

As the double labelling, virion density changing method, for detection of antibody described in the previous section appeared to be satisfactory in the model system with rabbit spleen and lymph node it was applied to cultures of RA synovial membrane.

Small fragments from 18 RA, 2 OA and 2 non-RA synovial membranes were incubated in the presence of ^{14}C-labelled amino-

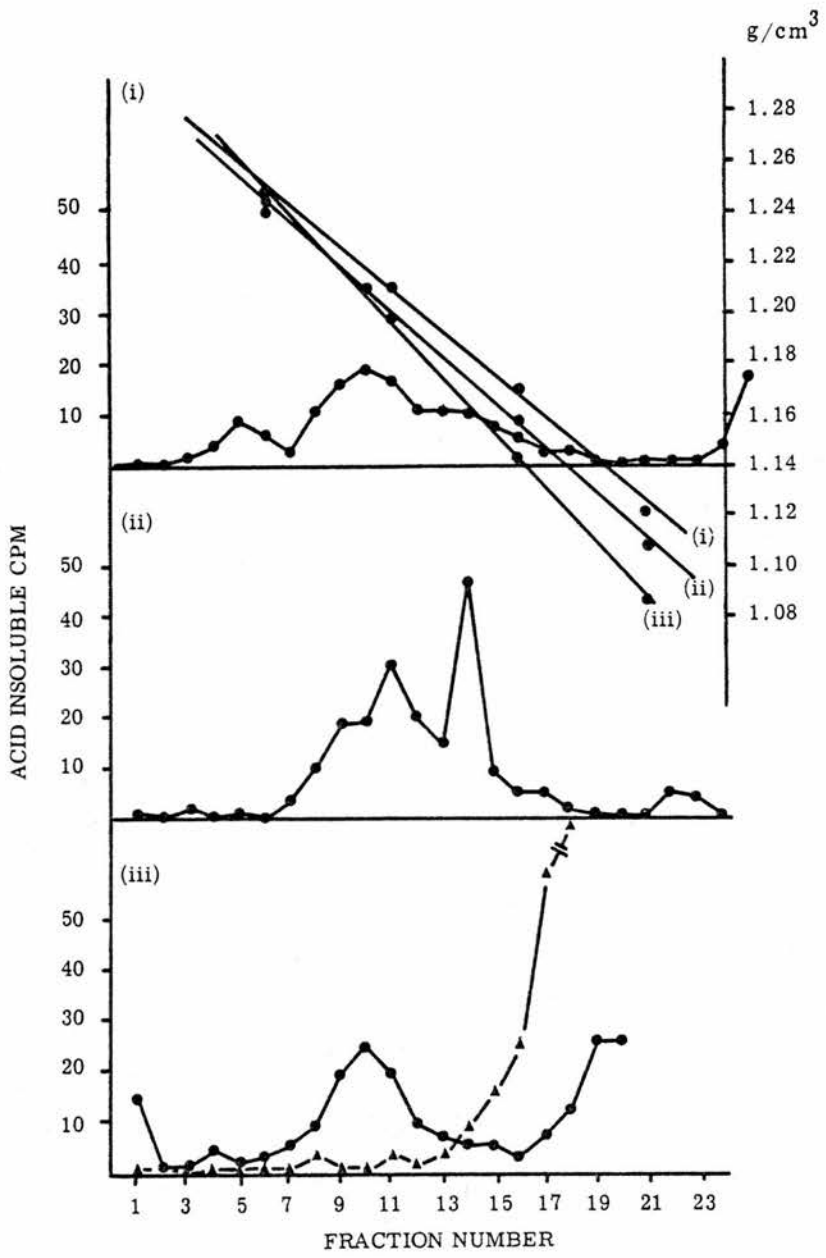


Fig. 15: Acid-insoluble cpm in sucrose density gradient fractions of (i) 100 µl of ^{3}H-uridine labelled measles virus mixed with 1/10 dilution of measles IgG

(ii) 100 µl of labelled measles virus only

(iii) 100 µl of labelled measles virus mixed with ^{14}C-labelled rubella antibody: (•—•) refers to ^{3}H-counts and (▲-▲) to ^{14}C-counts.

acids. The diagnosis and other criteria of these patients are listed in Table 5; the synovial membranes were obtained at the time of elective synovectomy or other surgery for therapeutic purposes.

Table 6 gives details of amounts of tissues and culture conditions. It can be seen that the initial attempts to produce labelled immunoglobulins failed in spite of substantial protein synthesis detected by the TCA precipitable counts. This failure was mainly attributable to the delay in transporting the specimens to the laboratory after their removal at operation. The situation was considerably improved by prompt delivery of the tissue specimens (specimen No's higher than 730020). Five out of eight fresh membranes incorporated the ^{14}C-label into immunoglobulins detected by immunoelectrophoresis and autoradiography. The immunoglobulins were of the IgG class, but some other labelled protein was also detected in the β_2 globulin region. This was not trailing human IgG as this band disappeared when the rheumatoid synovial supernatants were developed with monospecific anti-human IgG serum. No other bands have been seen in all the supernatants tested (Plate 7). A high, total TCA precipitable count ($\geq$ 200 cpm/10 μl), seemed a good indication of whether the specimen was likely to be positive for labelled immunoglobulins. It was conceivable that immunoglobulins might be synthesised by the synovial membrane lymphocytes in these cultures but then bound to antigens in the membrane and so not released into the fluid phase of the cultures. To test this possibility, acid or KSCN elution

Table 5

VRL No.	PMR case No.≠	Diagnosis	Sex	Age	Disease Duration	Tests for Rheumatoid Factor SSCT	Tests for Rheumatoid Factor Latex	ESR mm/hr	Drugs
720191	145622	OA	F	70	8 years	..	..	..	Indomethacin
720192	191295	RA	F	51	12 "	..	positive	82	..
720194	146300	RA	M	45	10 "	1024	..	50	Predinisolone Aspirin
720197	350310	OA	F	68	2½ "	..	..	1	Aspirin
720222	134607	RA	M	41	12 "	1/256	positive	59	Aspirin Chloroquine
720223	140665	RA	F	42	11 "	256	..	49	Aspirin Chloroquine
720226	104194	RA	F	57	16 "	..	positive	30	Aspirin Chloroquine
720227	135726	RA	F	59	22 "	..	positive	..	Aspirin
720234	145723	RA	F	53	11 "	..	positive	..	Aspirin Buprofen
720236	-	Knee trouble							
720241	146586	RA	M	62	2½ "	..	negative	..	Aspirin
720271	348710	RA	F	64	4 "	pos.	positive	27	..
720273	-	Ulnar neuritis	F	51	..	..	..	..	..
720274	144259	RA	F	72	13 "	128	..	63	Aspirin Codeine
730020	145387	RA	F	54	7 "	128	positive	20	Aspirin

VRL No.	PMR case No.≠	Diagnosis	Sex	Age	Disease Duration	Tests for Rheumatoid Factor SSCT	Tests for Rheumatoid Factor Latex	ESR mm/hr	Drugs
730026	141408	RA	F	59	5 years	..	positive	100	ACTH Butazolidine
730073	149185	RA	F	60	2 "	..	negative	40	..
730120	165290	RA	M	42	10 "	..	negative	..	..
730161	147146	RA	F	67	1½ "	..	..	72	Indocid Synthaethin
730167	220418	RA	F	65	4 "	1024	positive	84	Aspirin
730184	142136	RA	F	61	11 "	512	..	21	Aspirin Cortisone
740027	147136	RA	F	58	10 "	128	..	39	Solprin

≠ Patient number, Princess Margaret Rose Hospital.

.. = not tested.

TABLE 5: Clinical and other information on 20 patients with various forms of arthritis whose synovial membranes were used for in vitro synthesis of proteins.

Table 6

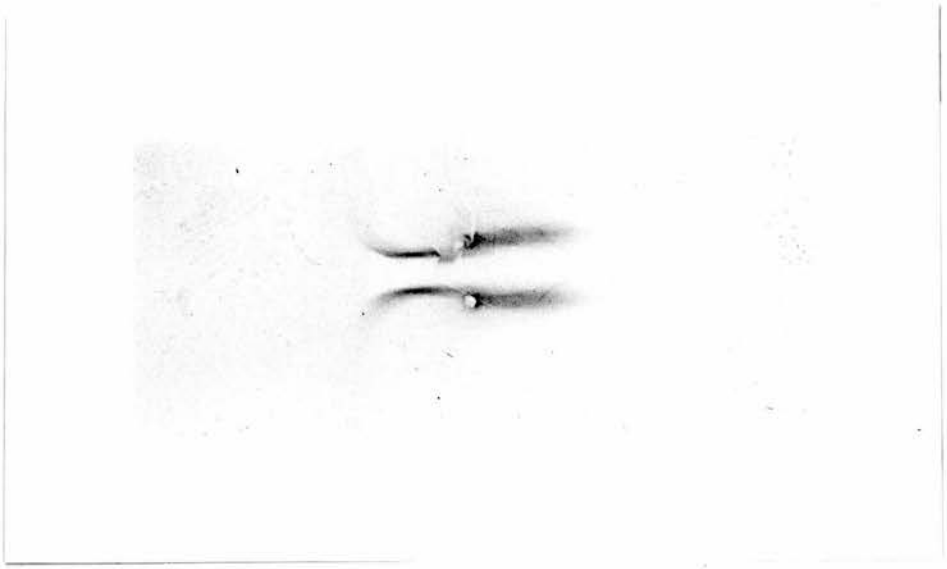
VRL No.	Net weight of specimen (g)	Medium added (mls)	Incubation time (hrs)	Radioactive label (mls)		TCA precipitable counts cpm/10 l	Ig (autoradiography)
				14C-lysine	14C-iso-leucine		
720191	0.42	1.3	6	0.01	0.01	7	-
720192	2.2	6.5	6	0.06	0.06	42	-
720194	1.5	4.3	6	0.04	0.04	1450	-
720197	2.2	6.5	7	0.06	0.06	104	-
720222	3.9	11.7	6	0.1	0.1	126	±
720223	1.5	4.4	24	0.04	0.04	1780	-
720226	1.6	4.6	24	0.04	0.04	833	-
720227	1.4	4.3	24	0.04	0.04	230	-
720234	1.0	3.0	24	0.06	-	940	-
720236	0.9	2.5	8	0.05	-	124	-
720241	1.5	4.5	24	0.04	0.04	2270	-
720271	1.8	5.5	24	0.06	0.06	1742	+
720273	1.5	4.4	24	0.04	0.04	32	-
720274	1.4	4.3	24	0.04	0.04	78	-
730020	1.0	3.0	16	0.03	0.03	746	+++
730026	1.6	4.8	16	0.04	0.04	196	-
730073	3.6	10.7	18	0.1	++	979	+++
730120	2.5	7.5	18	0.76 of	VLAL#	198	-

VRL No.	Net weight of specimen (g)	Medium added (mls)	Incubation time (hrs)	Radioactive label (mls)	TCA precipitable counts cpm/10 1	Ig (autoradiography)
730161	0.8	2.4	18	0.24 of VLAL	325	+++
730167	2.2	6.6	18	0.5 of VLAL	38	-
730184	2.3	6.9	18	0.6 of VLAL	281	+++
740027	4.8	14.4	18	0.7 of VLAL	..	++

≠ = collection of L-amino acids labelled with carbon-14 (Amersham CFB.103), 10 µCi/ml of solution; L-valine, L-leucine, L-arginine monochloride and L-lysine monochloride (2x).

.. = not tested.

TABLE 6: Background information and results of immunoglobulin synthesis by cultures of synovial membrane from 22 patients with various forms of arthritis.



Cathode
(-)

Anode
(+)

Plate 7: Immunoelectrophoresis with the supernatents from rheumatoid synovial membrane (central wells) cultured in the presence of ^{14}C-amino acids. The precipitation lines were developed with anti-human antiserum (trough) and allowed to sensitize film emulsion for 5 weeks.

treatments were performed with six of the membranes previously incubated in the presence of ^{14}C-amino acids. Table 7 shows the results. In two cases labelled immunoglobulins were eluted from the membrane. In one case (720222) the labelled eluate contained more labelled immunoglobulin than the fluid phase of the culture indicating binding with "antigen(s)" in the membrane. As the patient (720222) was also positive for rheumatoid factor (RF), this binding may have been due to locally synthesized RF reacting with the immune complexes deposited mainly in phagocytic lining cells, but other explanations cannot be excluded. Information about RF for 730161 was not available.

To test for antibodies against viral antigens the ^{14}C-labelled immunoglobulins were analysed by the sucrose density gradient method.

The group of viruses chosen for the experiments were rubella, measles, adenovirus (type 5) and feline leukaemia virus. The labelling and determination of the densities of rubella and measles virions is described above; the labelling of the adenovirus and feline leukaemia virus is now described.

Labelling of adenovirus 5 with ^{3}H-thymidine: One roller bottle (2.5 l) of HEp-2 cells was infected with an inoculum of adenovirus of unknown titre. One roller bottle of uninfected HEp-2 cells was kept as a control. An amount of 0.1 mCi of ^{3}H-thymidine was added to both the infected culture and control one day after the virus absorption, when the cytopathic effect was already starting.

Table 7

Specimen No.	Fluid phase of culture	Antibody elution from membrane fragments	
		Acid eluate	KSCN eluate
	Ig	Ig	Ig
720222	±	+	..
720271	+	-	..
730020	+++	..	..
730026	-	..	..
730073	+++	-	-
730120	-	..	..
730161	+++	..	±
730167	-	..	-
730184	+++	-	-
740027	++	..	..

+++, ++, +, -, = relative amounts of labelled immunoglobulin as detected by autoradiography

.. = not done

TABLE 7: Distribution of free and membrane bound immunoglobulins in experiments on the *in vitro* synthesis of proteins by synovial membrane fragments.

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Twenty-four hours later the fluid phase of the culture was concentrated by $(NH_4)_2SO_4$ precipitation and spun on a sucrose gradient (70% - 20%). Fig. 16 shows the result. Adenovirus is found at the high density of 1.25 - 1.26g/cm^3. As the density of an adenovirus - antibody complex was likely to be higher than this the percentage of sucrose in the gradients was increased to 80% - 20% w/v to check whether the complex would fall within the density provided by this gradient.

Volumes of 20 µl of ^{3}H-thymidine labelled adenovirus were mixed (i) with 180 µl of buffer (PBS pH 7.2, 0.01M) or (ii) with 100 µl of 1/5 dilution of a human serum containing complement fixing adenovirus antibodies and 80 µl of PBS buffer. The formation of antibody-virus complex (1 hour 37°C and overnight at 4°C) was tested by spinning on sucrose gradients (80% - 20% w/v). Fig. 17 shows a substantial increase in the ^{3}H-counts at the density 1.29 - 1.30g/cm^3 in (ii) compared to (i). Although all the counts were not removed from the viral density of 1.25g/cm^3, the formation of antibody adenovirus complexes was demonstrated and the density provided by 80% - 20% w/v gradients was sufficient to band the virus immune complex.

Labelling of feline leukaemia virus (FeLV) with ^{3}H-uridine:

FeLV virus chronically infected cultures of feline embryonic amnion (FEA) cells and the uninfected controls were examined by electron microscopy by Mrs A. Graham. Only one particle was seen in numerous cells examined (Plate 3). The cells were also

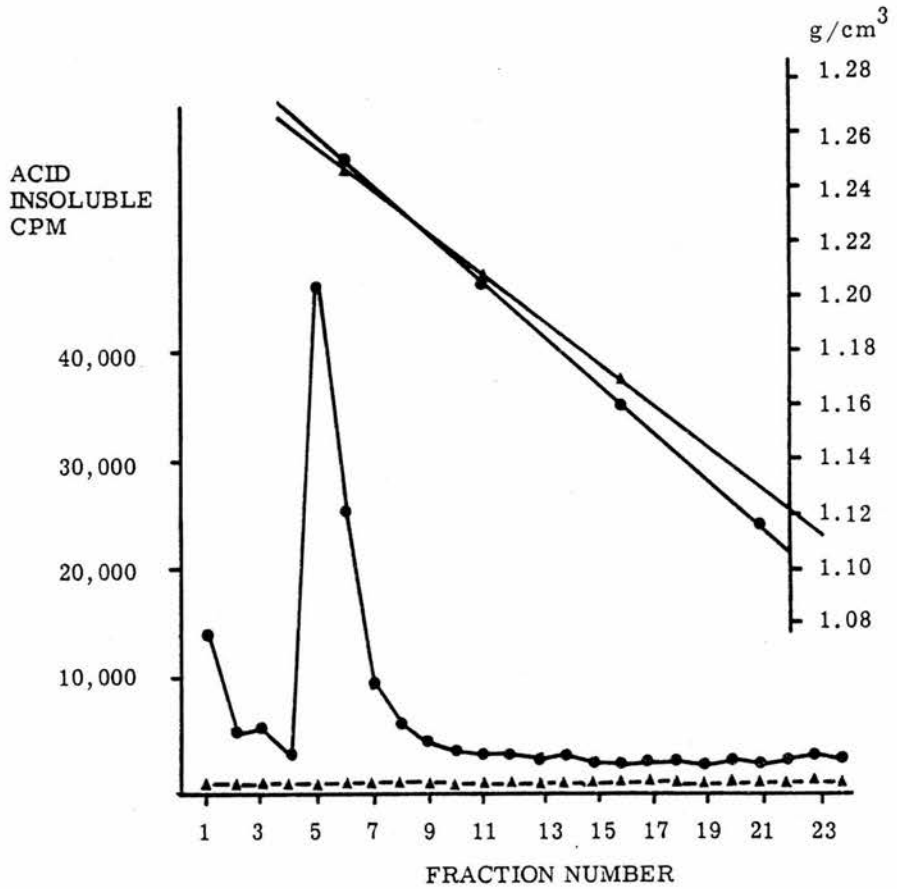

Fig. 16: Adenovirus infected H.Ep cells (•—•) and control HEp cells (▲-▲) labelled with 0.1 mCi of ^{3}H-thymidine.

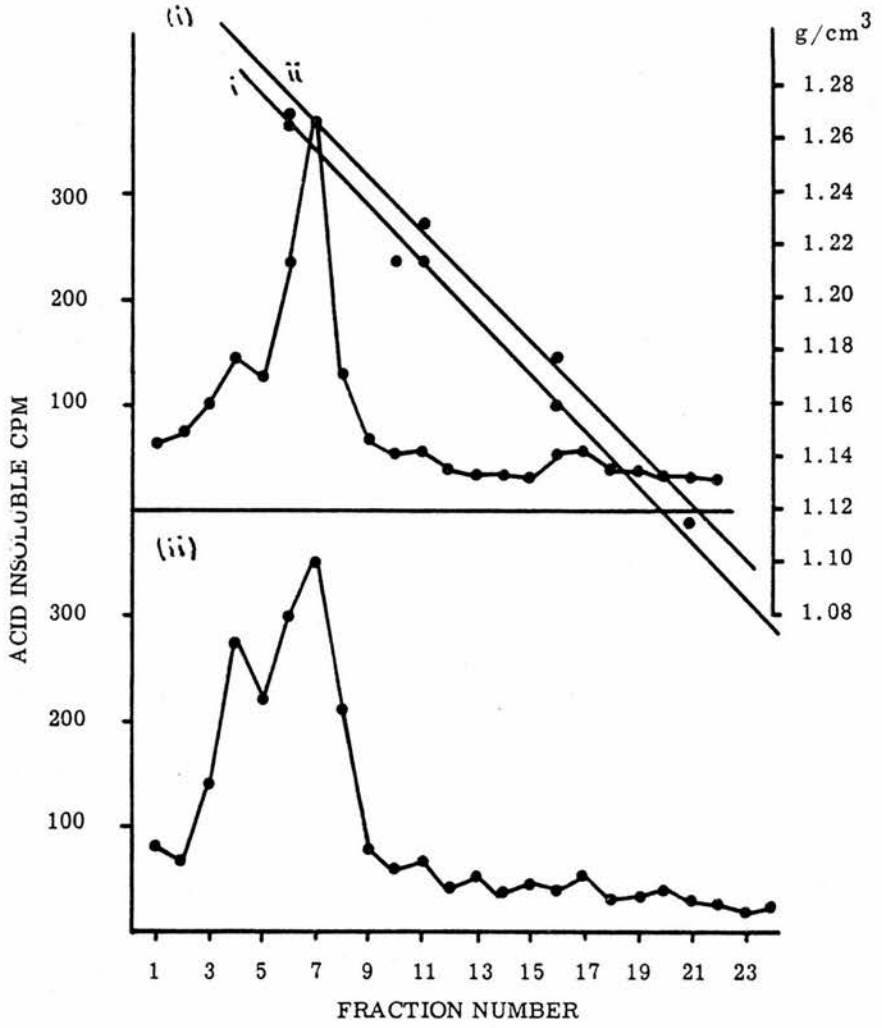

Fig. 17: (i) 20 µl of ^{3}H-thymidine labelled adenovirus spun on a sucrose gradient (80% - 20%)

(ii) 20 µl of ^{3}H-thymidine labelled adenovirus mixed with 100 µl of 1/5 human anti-adenovirus serum.

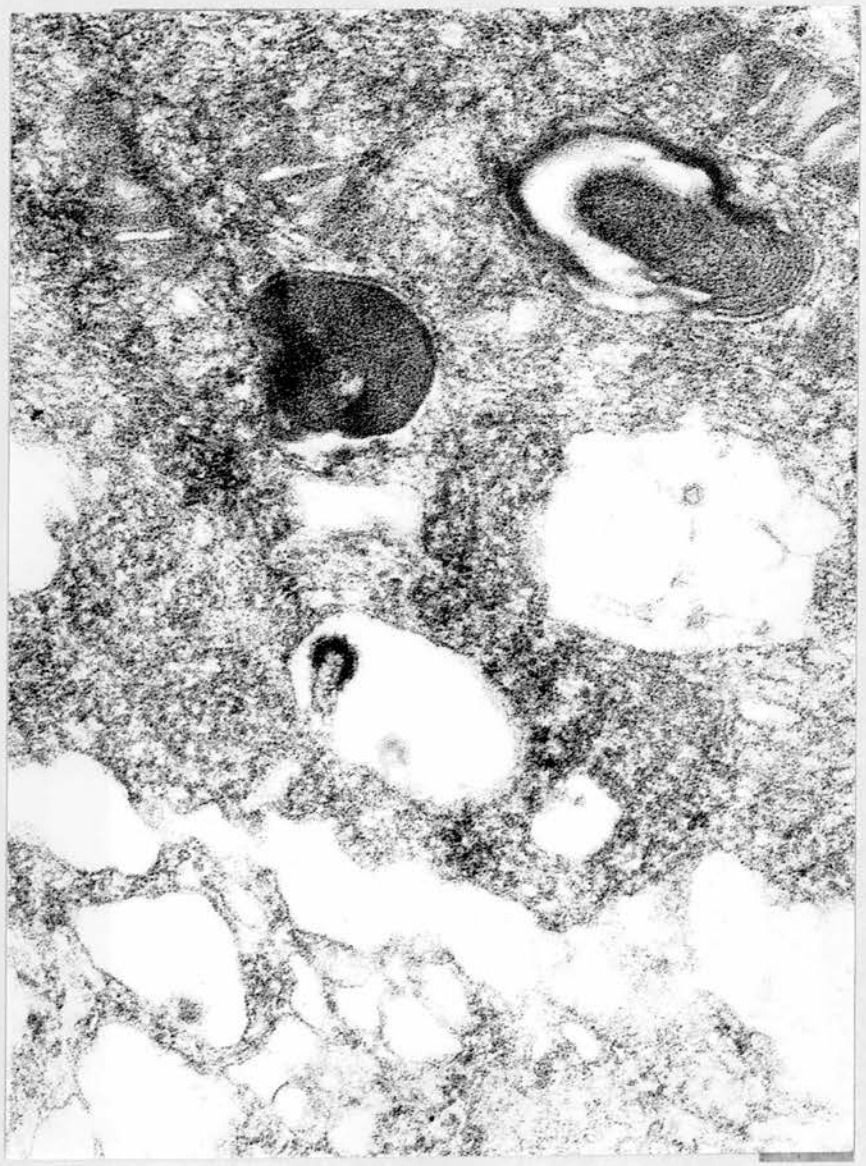

Plate 8: Electron microscopy examination of feline leukaemia virus infected feline embryonic amnion cells. Only one C-type virus was seen.

labelled with 0.1 mCi ^{3}H-uridine in two roller bottles. The fluid phase was precipitated and 0.1 ml analysed on a sucrose gradient (Fig. 18). The density of the virus in this system was 1.17g/cm^{3}; specific anti-FeLV sera were not available for analysis of the density of the complexes.

Sucrose gradient analysis of the synovial membrane immunoglobulins:

The four labelled viral antigen preparations were used at approximately 3,000 TCA precipitable counts per min (50 µl of rubella, 100 µl of measles, 10 µl of adenovirus and 200 µl of FeLV). These volumes and specific activities gave a viral peak on the sucrose gradient that contained 100-400 counts at the highest value. The ^{14}C-immunoglobulin preparations from synovial membranes were used at a concentration of 7,000 cpm. This number of counts had been used in the preliminary experiments with culture fluid phase from the rubella rabbit spleen-lymphnode preparation. A titration of the ^{14}C-immunoglobulins and the ^{3}H-viral antigens would have been more accurate to find the optimal concentration, but, as the main objective was to analyse the ^{14}C-synovial membrane immunoglobulins for the presence or absence of specific viral antibodies, no titration was carried out and the same number of ^{14}C-counts was used as in the rabbit spleen experiments.

The results of these experiments are set out in Table 9. It may be seen that the formation of virus-antibody complexes was not observed in most SM culture fluid phase preparations tested. Most of ^{3}H-counts remained at the viral density (1.18g/cm^{3} for rubella, 1.20g/cm^{3} for measles, 1.25g/cm^{3} for adenovirus and

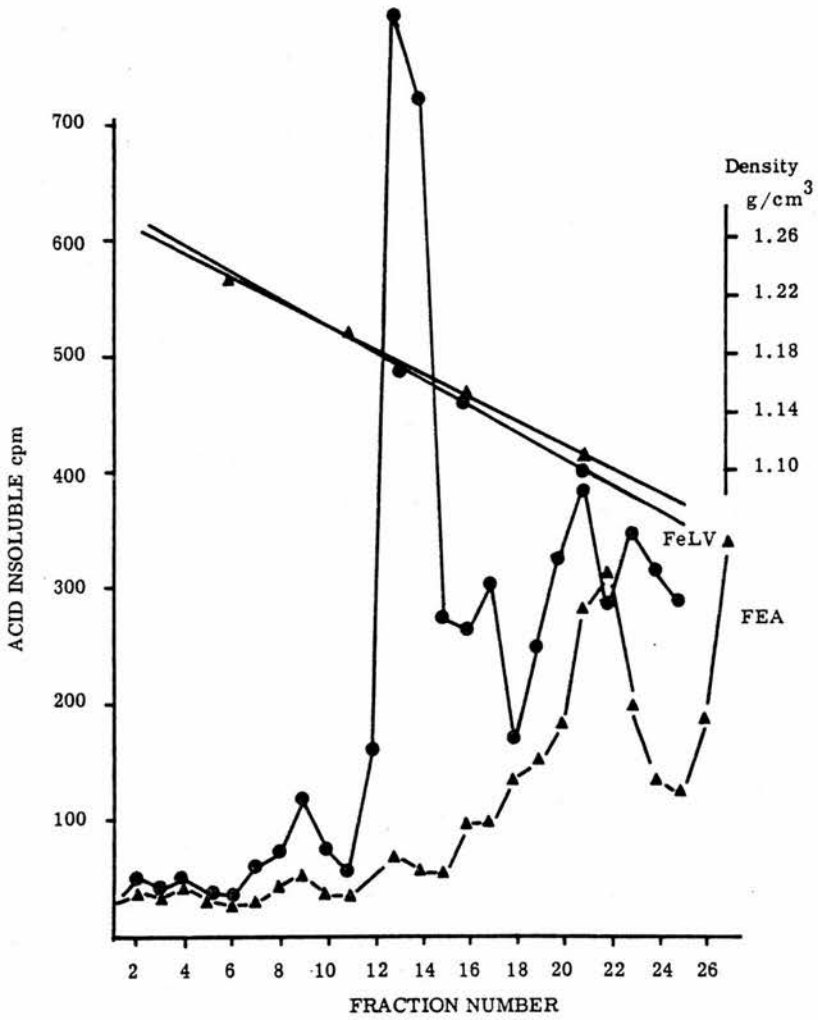

Fig. 18: Feline leukaemia virus infected FEA cells (●—●) and control FEA cells (▲-▲) labelled with ^{3}H-uridine and spun on sucrose gradients (70% - 20% in EDTA-Tris buffer, pH 7.2).

Table 9.

Rubella

virus density: 1.18-1.19g/cm^3

immune complex density: 1.25g/cm^3

Synovial membrane fluid phase		^{3}H-counts at 1.18-1.19 g/cm^3 (cpm)	^{3}H-counts at 1.25g/cm^3 (cpm)	^{14}C-counts at 1.18-1.19 g/cm^3 (cpm)	^{14}C-counts at 1.25g/cm^3 (cpm)
720222	a	40.3	442.1	0.0	19.0
720222	b	-	-	1.8	0.0
720271	a	411.7	7.9	10.9	0.0
720271	b	-	-	2.6	1.8
730073	a	273.9	28.0	7.3	12.3
730073	b	-	-	5.1	11.7
730161	a	207.7	6.4	7.1	0.0
730161	b	-	-	0.0	1.3
730167	a	22.9	200.4	0.9	2.4
730167	b	-	-	0.0	2.6
730184	a	221.0	28.4	1.0	2.3
730184	b	-	-	0.0	0.6
Rabbit spleen fluid phase	a	8.0	55.0	9.0	15.6
	b	-	-	6.2	0.0

Measles

virus density: 1.20-1.21g/cm^3

immune complex density: 1.24-1.26g/cm^3

Synovial membrane supernatant		^{3}H-counts at 1.20g/cm^3 (cpm)	^{3}H-counts at 1.25g/cm^3 (cpm)	^{14}C-counts at 1.20g/cm^3 (cpm)	^{14}C-counts at 1.25g/cm^3 (cpm)
720222	a	140.9	30.7	7.5	0.6
720222	b	-	-	0.9	3.9
720271	a	117.3	21.2	6.1	0.2
720271	b	-	-	1.3	1.8
730073	a	106.5	18.1	6.7	8.1
730073	b	-	-	5.6	11.7
730167	a	63.5	30.7	0.8	0.0
730167	b	-	-	0.0	1.5

Synovial membrane supernatant		^{3}H-counts at 1.20g/cm^3 (cpm)	^{3}H-counts at 1.25g/cm^3 (cpm)	^{14}C-counts at 1.20g/cm^3 (cpm)	^{14}C-counts at 1.25g/cm^3 (cpm)
730184	a	138.0	15.4	0.0	2.1
730184	b	-	-	0.0	0.0
740027	a	104.0	20.8	1.6	3.4
740027	b	-	-	14.6	1.2

Adenovirus

virus density: 1.25g/cm^3

immune complex density: 1.29g/cm^3

Synovial membrane supernatant		^{3}H-counts at 1.25g/cm^3 (cpm)	^{3}H-counts at 1.29g/cm^3 (cpm)	^{14}C-counts at 1.25g/cm^3 (cpm)	^{14}C-counts at 1.29g/cm^3 (cpm)
720222	a				
720222	b				
720271	a	123.6	11.3	8.5	1.7
720271	b	-	-	1.8	0.5
730073	a	61.2	109.8	9.1	1.1
730073	b	-	-	11.7	0.5
730167	a	141.5	9.0	3.1	0.1
730167	b	-	-	1.5	0.0
730184	a	126.3	6.8	0.0	0.0
730184	b	-	-	0.0	0.0
740027	a	114.6	105.8	0.0	2.2
740027	b	-	-	15.8	2.8

TABLE 9: The binding of ^{14}C-counts from in vitro labelled synovial membrane (SM) immunoglobulins in viral immune complexes: ^{14}C-labelled immunoglobulins were mixed (a) with 3H-viruses and complement or (b) with buffer for 1 hour at 37°C followed by overnight at 4°C. The mixtures were spun on sucrose gradients. 3H-counts indicate a distribution of the viruses: i.e., 3H-counts at the higher density show the formation of antibody-virus complex. ^{14}C-counts indicate specific anti-viral antibody binding in the complex.

1.17g/cm^3 for FeLV; latter not given in detail as the precise density of the immune complex was not known). However, a shift of ^{3}H-viral counts to the higher density characteristic of immune complexes occurred in two cases when the culture fluid phase preparations were mixed with rubella virus (namely, No's 720222 and 730167), and in one (No. 730073) in a mixture with adenovirus. However, only in one case (720222) was this shift in the density of the viral peak accompanied by increase of ^{14}C-counts at the density of immune complexes. This might indicate production of a specific rubella virus antibody by the synovial membrane of this particular patient. These results summarised in Table 10 include the observations with FeLV. Although the density of this immune complex was not known, no shift from the original FeLV viral density was observed with all RA membrane supernatants tested.

To further elucidate the importance of the single observation of rubella virus antibody produced by a RA membrane, acid eluates (glycine buffer pH 2.5) from 13 RA and one non-RA synovial membranes were also examined for antibody to rubella and measles by HAI. These were different clinical cases to those previously described. All were negative at dilutions of 1/2 - 1/4 (Table 11).

It seems that the immunoglobulins synthesised in the RA synovial membrane do not regularly contain high titres of antibody to rubella, measles or adenovirus. Nor was there evidence of antibody to feline leukæmia virus or retroviruses with related proteins.

Table 10.

Synovial membrane supernatant	Rubella	Measles	Adenovirus	FeLV
720222	++	-	..	..
720271	-	-	-	-
730073	-	-	-	..
730161	-	..	..	..
730167	-	-	-	-
730184	-	-	-	-
740027	..	-	-	-
Rabbit spleen	++	-	..	..

+ = positive binding of ^{14}C-immunoglobulins in the immune complexes

- = no binding

.. = not tested

TABLE 10: Summary of experiments on the binding of ^{14}C-counts from <u>in vitro</u> labelled synovial membrane immunoglobulins in antibody-virus immune complexes with ^{3}H virus and analysed on sucrose density gradients.

Table 11.

No.	PMR	NGH	Diag.	Measles HAI	Rubella HAI	OD 280
1A	165290	10106	RA	2	4	0.61
1B			10 years	2	4	0.82
2A	305127		RA	2	4	1.44
2B				2	4	0.48
3A		10040	RA	2	4	0.31
3B			10 years	2	NT	0.42
4A	221940		non-RA	2	4	0.62
4B				2	4	0.2
5A	144722		RA	2	4	
5B				2	4	
6B			3 RA tissues	2	4	
7A	220610		RA	2	4	0.52
7B			13 years	2	4	0.49
8A			RA	2	4	0.15
8B				2	4	0.44
9A	142134		RA	2	4	0.14
9B				2	4	0.51
10A	149990	15139	RA	2	4	0.26
10B			10 years	2	4	0.36
11A	107158	1625	RA	N.T.	4	0.98
			23 years		4	0.76
12A	147800		RA	N.T.	4	0.26
12B					4	0.49
13A	142120		RA	N.T.	4	0.9
13B					4	0.63
14A	147141	16376	RA	N.T.	4	0.9
14B					4	0.26

A: Last wash with PBS = 7.2

B: Eluate (pH = 2.5) from synovial membrane with pH adjusted to 7.2

NT: Not tested

TABLE 11: Synovial membrane eluates from patients with rheumatoid arthritis and other conditions as tested for viral antibodies.

RESULTS

RETROVIRUS STUDIES

Survey of sera from patients with rheumatoid arthritis for retrovirus antibodies

The evidence pointing to the possible involvement of retroviruses in some autoimmune states - particularly SLE - has been reviewed in the Introduction and interest has been reawakened recently by a paper from Philips, Sellers and Cotromei (1978).

In Edinburgh, the significance of retroviruses in RA has been studied by attempts to isolate virus from synovial fibroblasts or lymphocytes, by the examination of fibroblasts and lymphocytes for viral antigens and by examination of patients' sera for specific antibody. This section of the thesis describes work on the last two of these experimental approaches.

Several laboratories have reported the presence of antibodies in normal human sera that react with proteins of primate retroviruses (Snyder, Pincus and Fleissner, 1976; Aoki et al., 1976; Kurth et al., 1977) but others have failed to find such antibodies (Charman et al., 1974; Stephenson and Aaronson, 1976; Prochownik and Kirstein, 1976).

The retroviruses so far isolated from human tumours or blood cells comprise a mixture of two viruses, one closely related to simian sarcoma-associated virus (SSAV) and the other to the M7 strain of baboon endogenous virus (BEV) (Gallagher and Gallo, 1975). BEV virus is closely related to the endogenous feline virus RD-114

(Todaro et al., 1974). Although some doubt remains as to the origin and significance of these viruses, it is, nevertheless, reasonable to use them as reagents for the detection of viral antibodies, or antisera to them to detect their presence.

Antibodies specific to mammalian retroviruses in sera of patients suffering from rheumatoid arthritis and systemic lupus erythematosus, were compared with healthy controls. The antigens used in this study were RD-114 virus grown in RD-cells and SSAV grown in KNRK cells.

An antiserum from a rabbit immunised with RD-114 virus was absorbed with tissue and erythrocytes suspension (see Materials and Methods) and used to determine the optimal conditions for the solid phase radioimmunoassay. The anti-RD-114 serum after absorption differentiated clearly between RD-114 infected cells and control RD-cells. From Figure 19 can be seen that the values for the counts/100s bound to RD-114, after the deduction of the counts/100s bound to uninfected RD cells, were 1532 $\pm$ 189c/100s at the 1/300 serum dilution and 1124 $\pm$ 135c/100s at the 1/1000 serum dilution. The corresponding control serum gave negative values in both instances (-104 and -3c/100s). Sera positive for retrovirus antibodies can therefore be detected by the differential binding to the retrovirus infected and appropriate control cells. The same approach was used to examine the sera from patients with rheumatoid arthritis, systemic lupus erythematosus, from control subjects.

The sera obtained were divided into three groups.

Group I: contained sera from 10 RA patients; of these

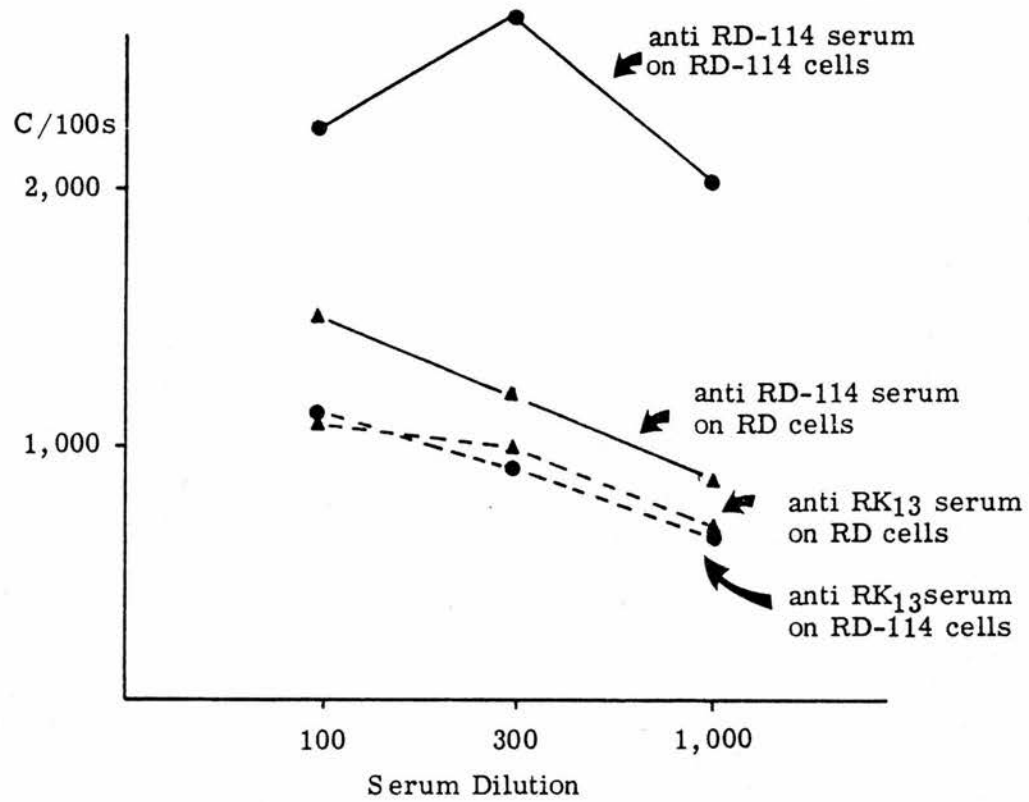

Fig. 19: The differential binding of the anti-RD-114 serum to the infected RD-114 RD cells and the uninfected RD cell control. The control anti-cell serum did not show this characteristic.

all were positive for RF, five showed ANF, two were negative for ANF and three were not tested.

Group II: a non-RA group with sera from five patients with OA, three with tennis elbow, one with polymyalgia rheumatica and one P.G.O.A. None was positive for either RF or ANF.

Group III: the sera in this group were from eleven SLE patients, all of whom had a positive ANF test and a negative RF test.

All sera were tested before and after absorption with insolubilised Cohn Fraction II to remove rheumatoid factor (see Materials and Methods).

The serum dilutions chosen for the study were 1/100, 1/300 and 1/1000. Figure 20 shows the distribution of values for the counts bound to RD-114 infected RD-cells, after subtracting the values for counts bound to uninfected RD cells. The distribution and range of the corrected values for radioactivity bound to the RD-114-RD cells was essentially the same in Groups I (RA) and II (Control) either before or after removal of rheumatoid factor. Sera in Group III (SLE) gave readings in a larger range of values than sera from RA and control subjects but some of the reactivity was removed by absorption for rheumatoid factor. This is a point of passing interest as Hannestad and Johannessen (1976) have shown cross reactivity between RF and ANF.

Figure 21 shows the results of titrating the three groups of sera on SSAV infected KNRK cells, both before and after absorption of RF. The patterns obtained were essentially similar

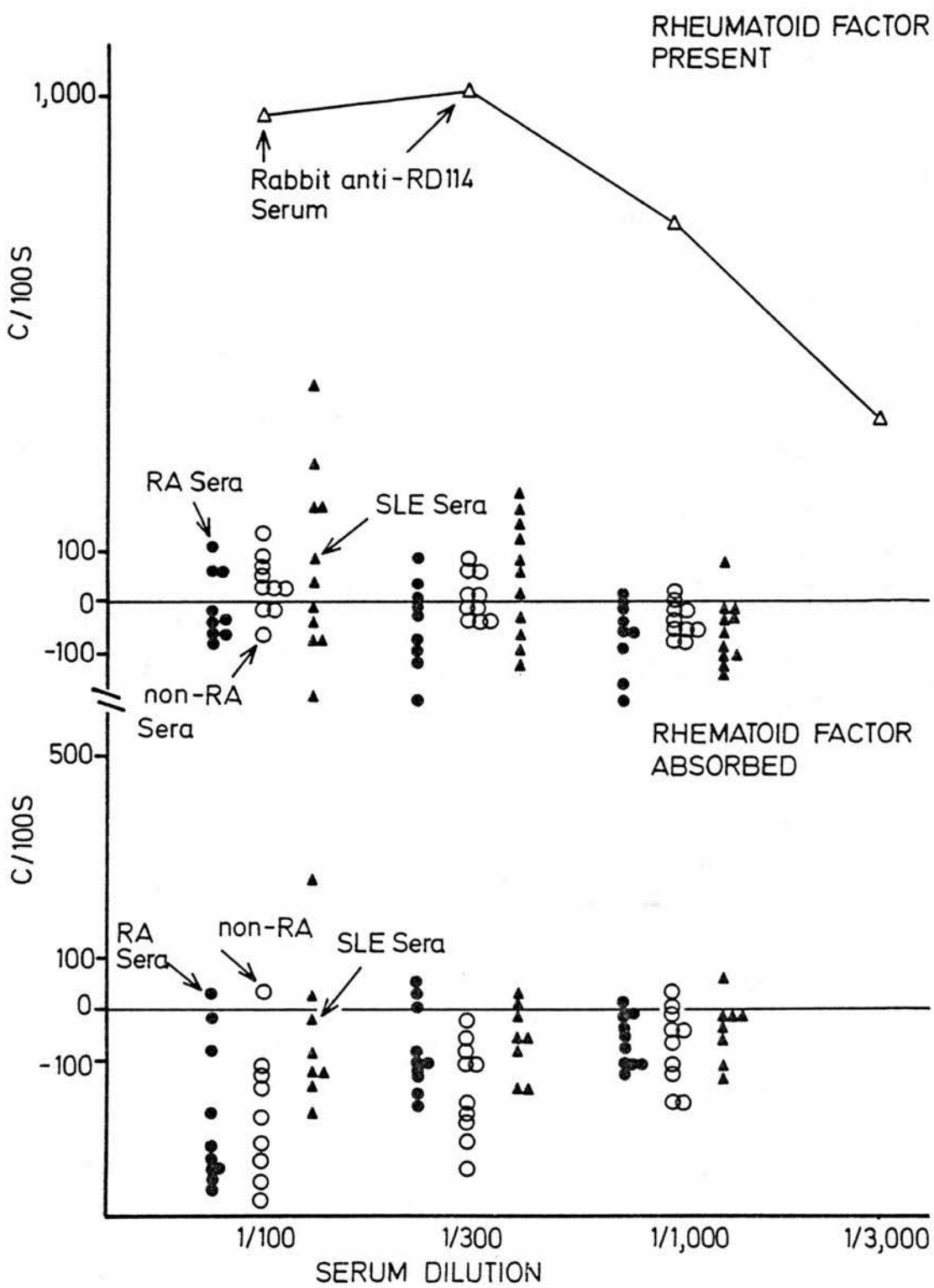

Fig. 20: The amount of Ig in human serum groups I, II and III bound in a solid phase radioimmunoassay with RD-114 virus infected after the values for bound globulin to uninfected RD cells were subtracted. Sera were tested before and after absorption with human serum Cohn fraction II. Symbols: ● = Group I, RA sera: o = Group II non-RA sera: ▲ = Group III SLE sera; △ = values rabbit anti-RD-114 antiserum.

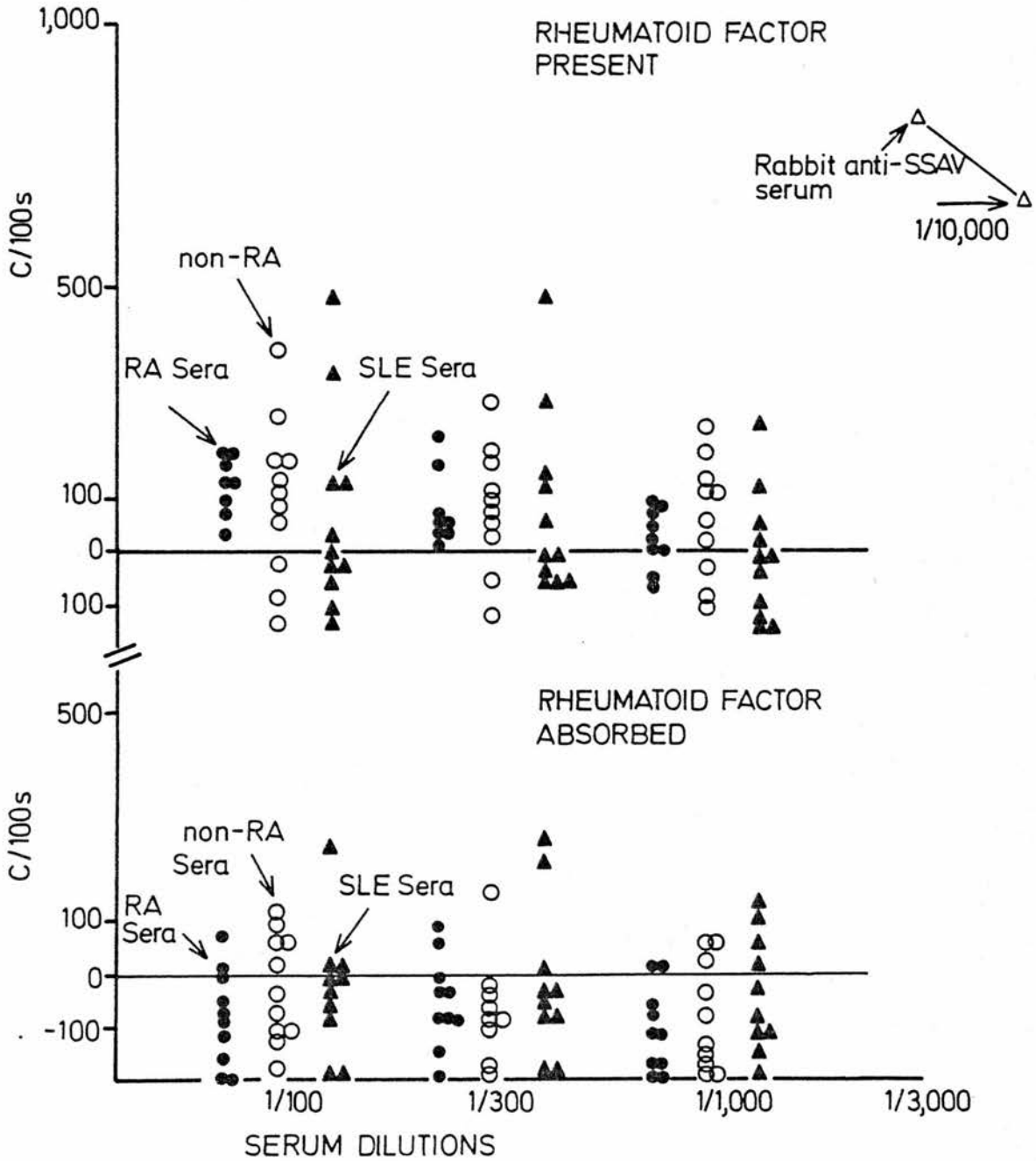

Fig. 21: The amount of Ig in human serum groups I, II and III bound in a solid phase radioimmunoassay to SSAV infected KNRK cells (KW 23 cell line) after subtraction of that bound to uninfected KNRK cells. Sera were tested before and after absorption with human serum Cohn fraction II.
Symbols: ● = Group I, RA sera; o = Group II, non-RA sera; ▲ = Group III, SLE sera; △ = values for rabbit anti-RD-114 antiserum.

to those with the RD-114 cells, perhaps with a slight overall elevation of the counts bound to the infected cells in all groups. Values with SLE sera (group III) had again a wider range than those from RA. The control sera from the hyperimmunised rabbits reacted satisfactorily: anti RD-114 titration end point 1/3000: anti SSAV 1/10,000.

These results do not provide evidence for retrovirus infection in RA. It is not possible to decide whether the (modest) amounts of globulin bound to the SSAV infected cells represent true antibody found in normal human sera. The removal of some reactivity after absorption with Cohn fraction II suggests non specific binding by RF.

Examination for retrovirus antigens on lymphocyte membranes

(a) Membrane immunofluorescence

A total of 22 samples of synovial fluid and 10 peripheral blood lymphocytes have been examined from patients with rheumatoid arthritis, psoriatric arthropathy or osteoarthroses. None of these showed membrane immunofluorescence with antisera obtained from the National Cancer Institute (NCI) directed against the Moloney mouse leukemia virus, Mason-Pfizer virus (p 12 + p 27), feline leukemia virus (p 12 + p 27), a commercial antiserum to Mycoplasma fermentans made in a goat and used as control for cross species reactions or normal goat serum. However, some batches of lymphocytes, particularly RA synovial lymphocytes, reacted with the NCI goat antisera to RD-114 and SSAV. The staining took the form either of a finely

granular deposit all round the periphery of the cell, or larger, intensely staining clumps at one pole of the cell suggestive of 'capping'. Table 11 sets out the intensity and the numbers of cells staining, by clinical diagnostic categories. The anti-RD-114 serum, which gave the most intense effect, stained not only most rheumatoid synovial fluid lymphocytes but also lymphocytes from patients with other conditions and from healthy individuals. In general, antigen positive lymphocytes were found consistently, but not invariable, to be present in larger numbers in RA synovial fluids, when compared with peripheral blood. The contrast was as much as 80% of positive lymphocytes in synovial fluid compared with 2% of lymphocytes from the peripheral blood of the same patient.

Various experiments were undertaken to establish the viral specificity of the reactions. Apart from antibody to viral antigens the antisera could contain antibody against other specificities RA synovial lymphocytes might present.

Examination of the viral antisera in gel-diffusion tests revealed antibody to calf serum but no reaction with human serum. Consequently, many lymphocyte cultures were done in human serum to avoid possible reactions with absorbed calf serum proteins. The membrane staining persisted. Low titre rheumatoid factor was detected in the goat antisera by agglutination of coated SRBC with human IgG. This was removed by adsorption with human serum-coated, glutaldehyde-fixed, sheep erythrocytes. This also did not alter the pattern of membrane staining; attempts to block membrane Ig by treatment with anti-human Ig serum before exposure to the

Table 12.

Condition	Lymphocytes	RD-114	MLV[1]	SSAV[2]	MPMV[3] (p12 + p27)	FeLV[4] (p12 + p27)
RA	SFL	13/15	1/15	10/15	0/15	0/15
	PBL	3/7	0/7	1/7	0/7	0/7
JRA	SFL	1/1	0/1	1/1	0/1	0/1
SLE	SFL	0/1	0/1	0/1	0/1	0/1
	PBL	0/1	0/1	0/1	0/1	0/1
Psoriatric arthropathy						
	SFL	2/2	0/2	1/2	0/2	0/2
	PBL	0/1	1/1	1/1	0/1	0/1
Osteoarthroses						
	SFL	1/2	0/2	0/2	0/2	0/2
Others: non-symptomatic						
	SFL	0/1	0/1	0/1	0/1	0/1
	PBL	1/1	0/1	0/1	0/1	0/1
Total	SFL	17/22	1/22	12/22	0/22	0/22
	PBL	4/10	1/10	2/10	0/10	0/10

1 = Moloney leukaemia virus
2 = Simian sarcoma associated virus
3 = Mason Pfizer mammary tumour virus
4 = Feline leukaemia virus
SFL = synovial fluid lymphocytes
PBL = peripheral blood lymphocytes

TABLE 12: Proportion of samples in various clinical categories stained with anti-retroviral sera by indirect membrane immunofluorescence. These antisera were obtained from National Cancer Institute (NCI). Positive reactions were observed with antisera to RD-114 and SSAV viruses.

viral antiserum were also ineffective.

Enquiry then revealed that the anti-viral sera (anti-RD-114 and anti-SSAV) that showed a strong positive reaction with human lymphocytes had been produced against viral antigens grown in cells of human origin (Table 12). Purified viral protein fractions had been used to immunise the goats but they might presumably have contained human cell surface determinants besides the viral antigens. It was noted that the Mason-Pfizer antiserum which did not react with RA synovial lymphocytes (Table 11) had also been produced against virus grown in a human lymphoblastoid cell line (NC 37); it was however conceivable that this virus matures without incorporating as many cell surface antigenic determinants.

The goat anti-RD-114 serum was then absorbed with human and sheep red blood cells four times to give a final dilution of the anti-retroviral sera of 1/5. After absorption sera lost their positive immunofluorescent staining with two pairs of rheumatoid synovial fluid and peripheral blood lymphocytes and reacted only faintly with RD cells infected with RD-114 virus; it is possible that the numerous absorptions required to remove the undesirable anti-"human" activity also removed most of the specific anti-viral antibody in the goat sera.

Goat anti-baboon C-type (batch 2) also cross-reacted with human antigens and 4 absorptions with human red blood cells were needed to remove the undesirable specificity, while anti-baboon type-C p28 appeared free of cross-reactivity measured by

Table 13.

	Virus or component	Batch No.	Species of animal immunised	Species of cell used for propagation of virus
(1)	Baboon Type C virus tween ether disrupted	4S-504	Goat	Baboon kidney-canine thymus co-cultivated culture(chiefly canine thymus)
(2)	Baboon Type C virus p28	5S-463	Goat	Same as (1)
(3)	Woolly monkey fibro-sarcoma virus (SSV-1) tween ether disrupted	3S-172	Goat	Human (NC-37) lymphoblastoid line
(4)	RD-114 virus tween ether disrupted	3S-73	Goat	Human rhabdomyo-sarcoma line
(5)	RD-114 virus p28	2S-781	Goat	Same as (4)
(6)	Feline leukæmia virus p27 and p12	2S-65	Goat	Feline cells (FL-74)
(7)	Mason-Pfizer Monkey mammary tumour virus, p.27 and p12	4S-517	Goat	Human (NC-37); same as (3)
(8)	Moloney leukæmia virus tween ether disrupted	1S-160	Goat	Mouse(NIH 3T3)

TABLE 13: Species of cells used for the propagation of the viral antigens prior immunization of goats (information supplied by Dr. J. Gruber, NCI, Bethesda).

agglutination of HRBC. The baboon antisera free of 'normal human antigen' cross-reactivity failed to stain 2 pairs of rheumatoid synovial and peripheral blood lymphocytes.

In further attempts to elucidate the difference in the immunofluorescence patterns observed with rheumatoid synovial fluid (SFL) and peripheral blood lymphocytes (PBL), new antisera were raised in rabbits. The RD-114 virus was adapted to growth in rabbit kidney (RK13) cells grown in rabbit serum. The SSAV was grown in KNR cells, cultured in rabbit serum. The partly purified virus from these cultures was then used as an inoculum for the rabbits. Similar fractions from uninfected cells were inoculated into rabbits to provide control sera. Table 13 shows the results testing synovial and peripheral blood lymphocytes with these antisera. All lymphocytes were negative with anti-RD-114 serum and its control serum. Anti-SSAV serum and control serum stained 1-2% of cells, but the majority were negative. Satisfactory staining was obtained with RD-114 infected control cells.

The study was extended by trypsinizing the lymphocytes, in particular those from the synovial fluid, before immunofluorescent staining. Trypsin treatment is reported to remove immune complexes that block the receptors for sheep erythrocytes on T-lymphocytes from RA patients (Wangel and Klockars, 1977); such complexes might conceivably mask retrovirus antigen. Nevertheless, all immunofluorescent tests were negative with specific anti-viral sera, including two samples of lymphocytes isolated from rheumatoid synovial membranes rather than harvested from SF.

The negative results by immunofluorescence were checked by

Table 14

VRL No.	Diagnosis	Lymphocytes	Retrovirus antisera			
			R35/RD-114RK$_{13}$/6 -29.8.77	R33/RK$_{13}$/6 -29.8.77	R42/KW23/4 -9.12.77	R40/KNRK/4 -9.12.77
770071	RA (14 years)	SFL	-	-	..	..
		PBL	-	-	..	..
770098	RA (24 years)	SFL	-	-	..	..
		SFLTR	-	-	..	..
		PBL	-	-	..	..
770102	Seroneg RA (2 years)	SF	-	-	..	..
		SFTR	-	-	..	..
		PBL	-	-	..	..
		PBLTR	-	-	..	..
770099	RA (13 years)	SMTR	-	-	..	..
770100	RA (7 years)	SMTR	-	-	..	..
770108	RA (9 years)	SF	-	-	..	..
		SFTR	-	-	..	..
		PBL	-	-	..	..
770109	RA (20 years)	SF	-	-	..	..
		SFTR	-	-	..	..
		PBL	-	-	..	..
770144	RA (8 years)	SF	-	-	±	±
		SFTR	-	-	..	..
		PBL	-	-	±	±

VRL No.	Diagnosis		Lymphocytes	Retrovirus antisera			
				R35/RD-114RK$_{13}$/6 -29.8.77	R33/RK$_{13}$/6 -29.8.77	R42/KW23/4 -9.12.77	R40/KNRK/4 -9.12.77
770148	RA (20 years)		SF	–	–	±	+
			SFTR	–	–	±	±
			PBL	–	–	..	..
770149	RA (24 years)		SF	–	–	..	..
			SFTR	–	–	..	..
			PBL	–	–	..	..
770122	RA (15 years)		SF	..	..	–	–
			PBL	..	..	–	–
770123	RA (6 years)		SF	..	..	–	–
			PBL	..	..	–	–
770164	? early RA (1 year)		SF	..	..	±	±
			PBL	..	..	±	±
770004	RA (15 years)		SF	..	..	±	±
			PBL	..	..	±	±
	Normal	I	PBL	–	–	±	±
		II	PBL	–	–	±	±
NC-37 Human lymphoid cell-line				..	..	±	±
SSAV - No.37				..	..	+++	±
RD-144 infected RD cells				+++	–	..	..
RD cells				–	–	..	..
KW 23				–	–	++*	±*
KNR				–	–	–*	+*

TABLE 14: Distribution of membrane immunofluorescence with rabbit antiretroviral sera to RD-114 (R35/RD-114 RK$_{13}$/6) and to SSAV viruses (R42/KW23/4). In this

study controls included sera produced against uninfected cells (R33/RK_{13}/6 and R40/KNRK/4.) No specific binding was observed to rheumatoid and other lymphocytes irrespective of the trypsin treatment.

Symbols: +++, ++, +, ± = various degrees of immunofluorescence;

– = negative result; .. = not tested;

* – the immunofluorescence was obtained only after numerous absorptions with rat liver powder and KNRK cells and thus differed from the above samples where only a mixture of HRBC and SRBC was used.

radioimmunoassay and complement mediated cell cytotoxicity as alternative methods of detecting cell antigens.

(b) Radioimmunoassay. Nine samples of trypsinized cells from synovial fluid and twelve cultured sets of lymphocytes all with matched peripheral blood lymphocytes were tested by radioimmunoassay for the binding of antibodies to RD-114 and SSAV. In control experiments RD-114 infected cells showed substantially higher binding ratios with anti-viral sera (calculated as 2.75 at serum dilution 1/100; 2.89 at serum dilution of 1/300) than with sera inoculated with cells alone (R33/RK13/6 - 29.8.77 and R40/KNRK/4 - 9.12.77) (Figure 22). The trypsinized and untreated synovial fluid lymphocytes, and peripheral blood lymphocytes and uninfected RD cells, did not show any binding at the dilutions of 1/100 - 1/300 with either of the antisera (Table 14).

(c) Complement-mediated cell cytotoxicity.

After the completion of the tests on lymphocytes by immunofluorescence and radioimmunoassay, Oliver and Pillai (1977) reported that retrovirus antigens on the surface of leukaemic cells could be detected by complement-mediated cell cytotoxicity. Particularly, goat anti-SSAV serum reacted with lymphocytes from patients with acute lymphocytic leukaemia. It was conceivable that this technique might, for reasons of sensitivity or different mechanism, reveal antigens that the other two techniques had not detected. Cell viability was detected by a dye-exclusion method. The cytotoxic activity of the antiserum to RD-114 was determined by

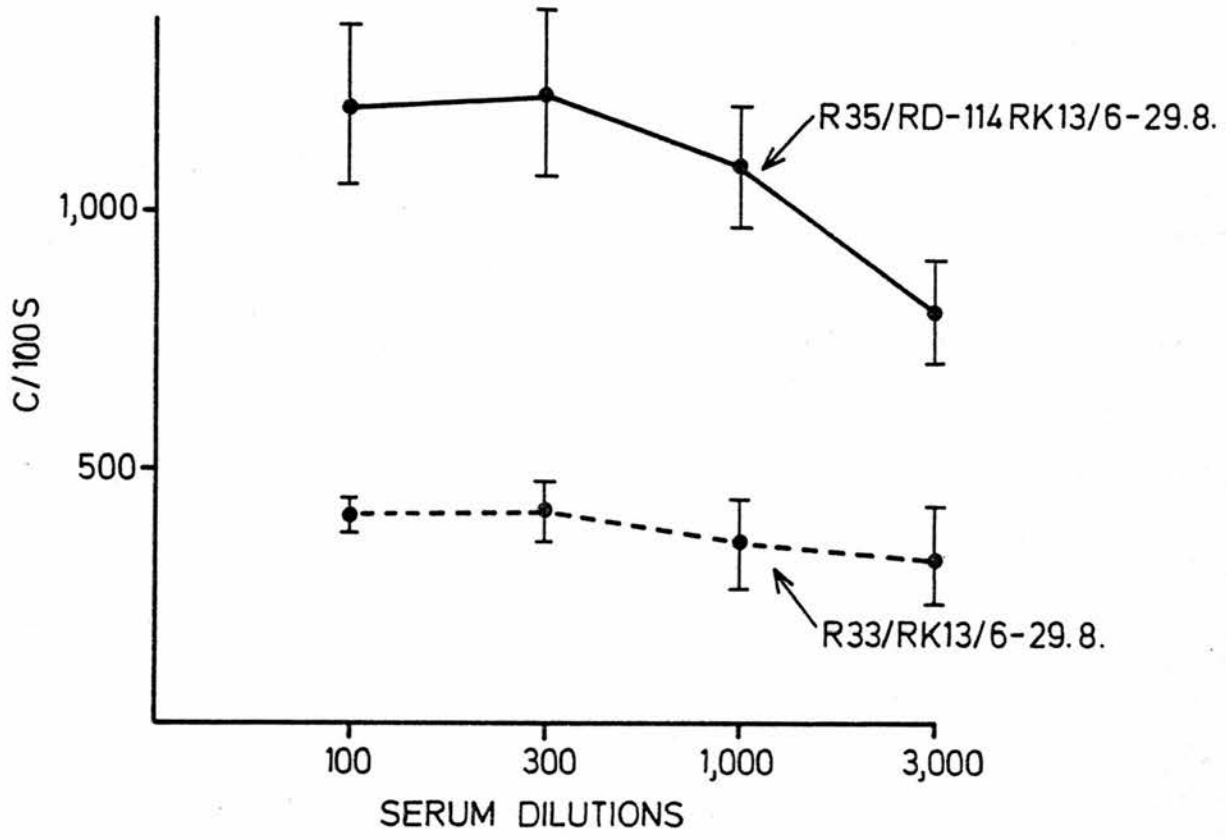

Fig. 22: The differential binding of anti-RD-114 serum (R35/RD-114 RK_{13}/6-29.8) and control anti-"cell" serum (R33/RK_{13}/6-29.8) to RD-114 infected RD cells. Each point represents the mean calculated from three observations. The standard deviation is indicated by a horizontal line at each serum dilution.

Table 15

VRL No.	Diagnosis	Lymphocytes	Binding ratio $\frac{R35/RD\text{-}114RK_{13}/}{R33/RK_{13}/}$ at serum dilutions 1/100	1/300	1/1,000	$\frac{R42/KW23/4\text{-}9.12.77}{R40/KNRK/4\text{-}9.12.77}$ at serum dilutions 1/100	1/300	1/1,000
770102	Seroneg RA (2 years)	SFL	1.19	0.94	..	..	..	..
		SFLTR	1.07	1.42	..	..	..	..
770108	RA (9 years)	SFL	..	0.98	..	..	..	..
		SFLTR	..	0.90	..	..	..	..
		PBL	..	1.32	..	..	..	..
770109	RA (20 years)	SFL	..	0.90	..	..	..	..
		SFLTR	..	0.48	..	..	..	..
		PBL	..	0.71	..	..	..	..
770132	RA (26 years)	SFL	..	0.97	..	..	..	..
		SFLTR	..	0.60	..	..	..	..
		PBL	..	1.08	..	..	..	..
770134	RA (3 years)	SFL	1.47	0.82	..	..	..	..
		SFLTR	1.05	0.91	..	..	..	..
		PBL	..	0.88	..	..	..	..
770144	RA (8 years)	SFL	0.62	0.77	..	..	..	..
		SFLTR	0.55	0.77	..	..	..	..
		PBL	0.39	0.52	..	..	..	..
770148	RA (20 years)	SFL	..	1.42	..	..	1.25	..
		SFLTR	..	0.93	..	..	..	..
		PBL	..	1.11	..	..	0.96	..

VRL No.	Diagnosis		Lymphocytes	Binding ratio $\frac{R35/RD\text{-}114RK_{13}/}{R33/RK_{13}/}$ at serum dilutions 1/100	1/300	1/1,000	$\frac{R42/KW23/4\text{-}9.12.77}{R40/KNRK/4\text{-}9.12.77}$ at serum dilutions 1/100	1/300	1/1,000
770149	RA (24 years)		SFL	..	1.40	..	..	..	..
			SFTR	..	1.15	..	..	..	..
			PBL	..	1.16	..	..	..	..
770152	JRA (16 years)		SFL	0.78	..	..	..	..	..
			SFTR	0.72	..	..	..	..	..
			PBL	3.47	..	..	..	..	..
770122	Seroneg RA (15 years)		SFL	..	..	..	..	0.58	..
			PBL	..	..	..	..	0.63	..
770123	RA (6 years)		SFL	..	..	..	..	0.97	..
			PBL	..	..	..	..	0.85	..
770164	? early RA (1 year)		SFL	..	..	..	..	0.81	..
			PBL	..	..	..	..	1.72	..
	Normal	I	PBL	1.53	..	..	0.95	..	..
		II	PBL	1.71	..	..	1.12	..	..
RD-114 infected cells				2.75	2.89	2.83	..	..	..
RD cells				1.10	1.12	0.87	..	..	..

.. = not tested

SFL = synovial fluid lymphocytes
SFLTR = trypsinized synovial fluid lymphocytes
PBL = peripheral blood lymphocytes

TABLE 15: Detection of retrovirus antigens by indirect radioimmunoassay on rheumatoid synovial and peripheral blood lymphocytes.

titration on infected and uninfected RD cells, with and without rabbit complement. The 'anti-cell' serum (R33/RK13/6-28.8.) was included as a further control. The results are shown in Figure 23; there was a clear cut difference between specific antigen and controls. It will be noted that the 50% cytotoxicity was produced by a dilution of 1/40 of the RD-114 antiserum - this contrasts with a titre of at least 1/3000 obtained by radioimmunoassay (Figure 22). As a result of these titrations, a dilution of 1/20 of the RD-114 antiserum was chosen for cytotoxicity tests with synovial (SFL) and peripheral blood lymphocytes (PBL) from six RA patients, together with PBL from three healthy persons. Four batches of RA-SFL were treated with trypsin before testing (=SFLTR). Three other pairs of RA-SFL and PBL, plus three lots of PBL from healthy persons were tested with a 1/20 dilution of anti-SSAV serum.

The levels of cytotoxicity observed with both of the viral antisera were low with all batches of lymphocytes and did not reveal any trend unobserved by the other techniques already employed (Figure 24). With the anti-RD-114 serum the mean values for RA-PBL, SFL and SFLTR were 4.8%, 6.1% and 1.8% respectively, compared with 62% for the antiserum on RD-114 infected RD cells, and 0% of normal PBL and uninfected RD cells. It was noted that the untreated synovial fluid lymphocytes were more fragile and the background levels of dead cells were consistently higher than those found in preparations of peripheral blood lymphocytes from the same patient. This observation fits with the leakage of cell macromolecules from RA-SL noted in other work (Norval, personal

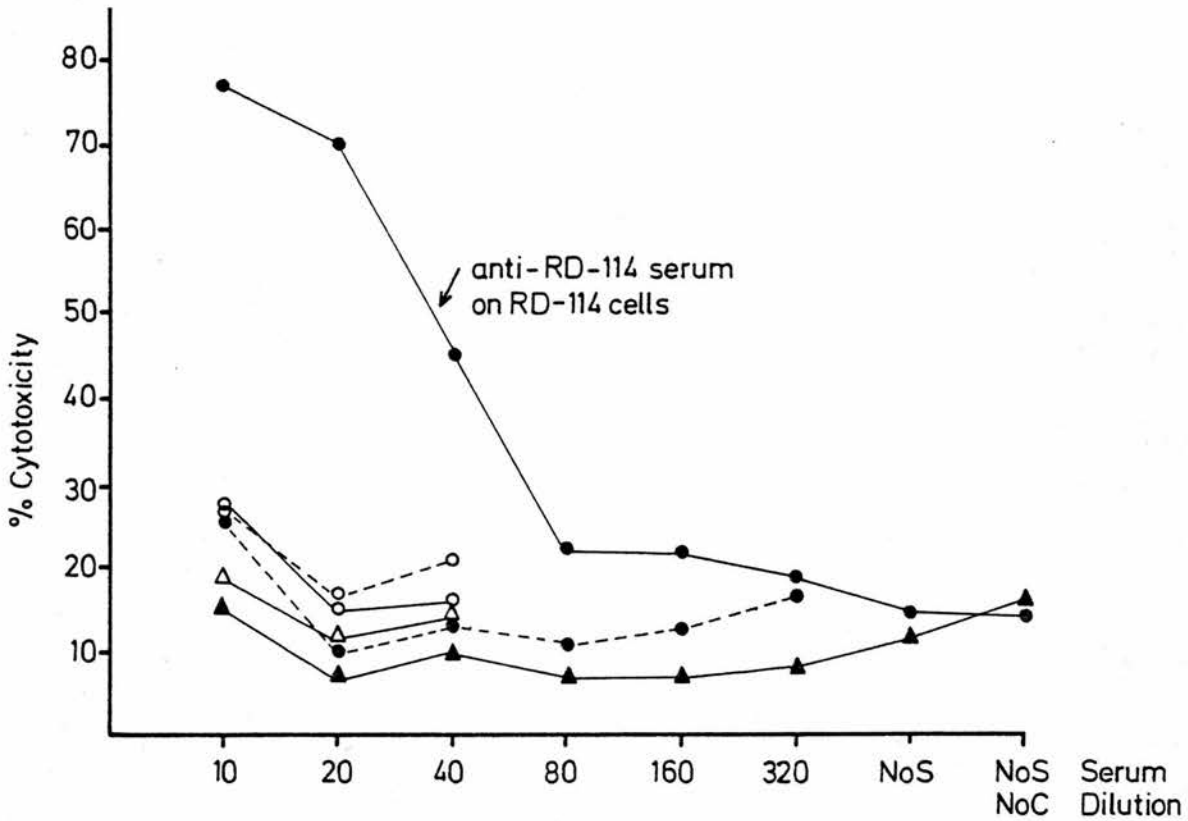

Fig. 23: Cytotoxicity by anti-RD-114 serum (—R35/RD-114 RK_{13}/6-9.8) and anti-cell control serum (---R33/RK_{13}/6-29.8) on RD-114 infected (●) and control RD cells (▲) in the presence (●; ▲) and absence of complement (○; △).

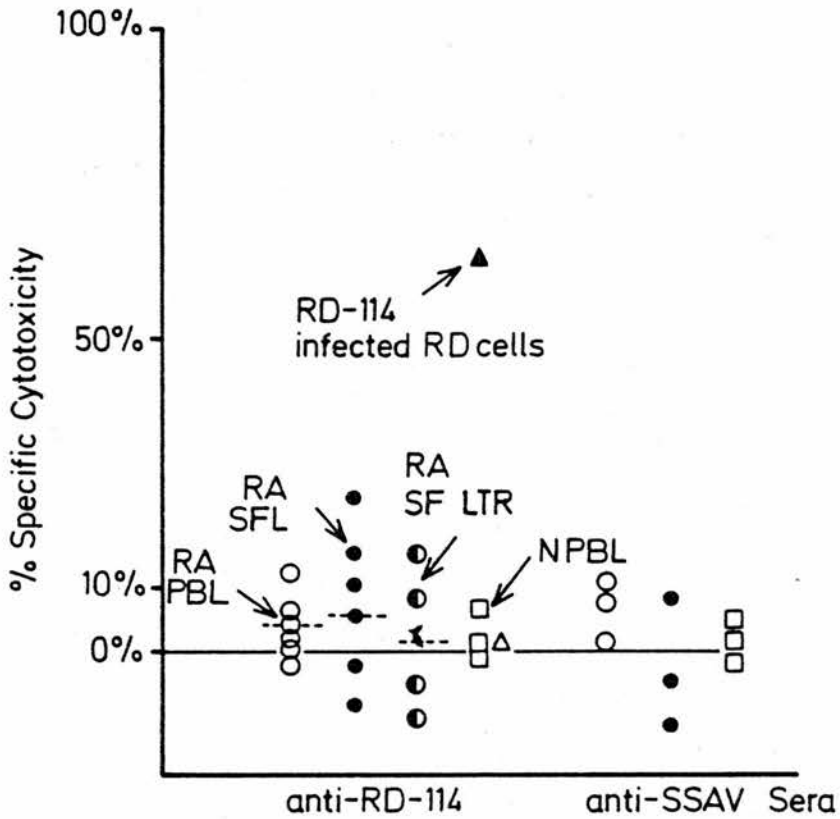

Fig. 24: Examination of retroviral antigen specific complement-mediated cytotoxicity using rheumatoid peripheral blood lymphocytes (RA PBL = O), rheumatoid synovial blood lymphocytes obtained from the same patients on the same day (RA SFL = ●), trypsinized rheumatoid synovial fluid lymphocytes (RA SFLTR = ◐), normal peripheral blood lymphocytes (NPBL = □). Controls included RD-114 infected RD cells (▲) and uninfected RD cells (△).

communication).

Search for retrovirus antigens in sections of synovial membrane

It was clear from the foregoing work that antigens related to primate, murine and feline retroviruses are not present in significant amounts on RA synovial lymphocytes. Other work (see Introduction for references) has not revealed retrovirus reverse transcriptase in RA synovial membrane extracts or synovial fibroblast cultures. It seemed important, nevertheless, to examine sections of synovial membrane that would include cells not represented in suspensions of synovial lymphocytes or fibroblast cultures. (A possible analogy was the claim by Panem et al., 1976, to have demonstrated retrovirus antigens in the mesangial cells and along the glomerular capillary walls in SLE kidneys).

Accordingly, fixed frozen sections and dewaxed paraffin sections from five RA and five non-RA synovial membranes were examined, respectively, by immunofluorescence and immunoperoxidase techniques, with the anti-RD-114 and SSAV sera and their corresponding control 'anti-cell' antisera, and also with antisera to IgG, IgM and C3 (Table 15). Immunofluorescence or immunoperoxidase staining was not observed with the viral or the control antisera. As would be expected, bound complement and immunoglobulin was detected, particularly in the RA membranes and particular attention was paid to these areas when looking for viral specific staining. There was less staining by the anti-

Table 16.

No.	Diagnosis	Antiserum IgG	IgM	C3	RD-114	C	SSAV	C
IF 1	RA (15 years)	-	+++	+	±	-	-	-
2	RA (25 years)	-	++	+++	-	-	-	-
3	OA	+	++	++	-	-	-	-
4	OA (5 years)	-	±	++	••	-	-	-
5	OA (3 years)	-	-	+	-	-	-	-
6	Early degeneration of knee (1 year)	++	-	+	-	-	-	-
IP 7	RA (12 years)	±	++	+	-	-	-	-
8	RA (seronegative 20 years)	++	-	-	-	-	-	-
9	RA (4½ years)	++	+	-	-	-	-	-
10	OA (8 years)	-	-	++	-	-	-	-

C = anti-cell sera

TABLE 16: Detection of serum proteins and retrovirus antigens by indirect immunofluorescence (IF) and immunoperoxidase (IP) in, respectively, frozen and paraffin sections of synovial membranes from patients with various diagnoses. Sera used in this study were identical to those previously described (see Table 13).

IgG serum than might have been predicted. 'Masking' IgM RF might have to be removed to increase this, but it was not attempted.

DISCUSSION

The Discussion is arranged in sections corresponding to the four general areas of work described under Results.

Rubella and rheumatoid arthritis

Cultured synovial lining cells type B and C have been extensively investigated for the presence of vegetative virus particles by techniques that would detect a productive rubella virus infection, but with negative results (Person, Sharp and Rawls, 1973; Norval and Marmion, 1976). It is conceivable that an association of rubella virus and synovial fibroblasts, without liberation of virions but with expression of antigens on the cell surface, might occur; this possibility was suggested by the results of Patterson, Howard and Deinhardt (1973). The viral neo-antigens expressed on these cells might provide a continuous stimulus for the patient's immune system, especially if, due to a deficiency in cell-mediated immunity, the altered cells were not eliminated.

The ability of rubella virus to persist in cells is well known from congenital rubella infection (Rawls, 1968) and from observations of carrier cultures, such as LLC-MK_2 cells (Brown et al., 1964) and other chronically infected continuous cell lines (Stanwick and Hallum, 1974). In addition, lymphocytes from congenital rubella cases (Simons and Fitzgerald, 1968) and rabbit embryonic chondrocytes (Smith et al., 1973) may be persistently infected. The chronically infected rubella LLC-MK_2 cell line

established in our laboratory shows characteristics typical of rubella virus persistence. It produced infectious virions for up to one year and these could be detected by the incorporation of ^{3}H-uridine into particles of density 1.18g/cm^{3} as well as by subculture into a sensitive cell line allowing a cytopathic effect, for example RK_{13} cells. The chronically infected LLC-MK_2 cells were readily and specifically stained for cytoplasmic antigen by immunofluorescence when acetone fixation was used, but membrane antigens were not demonstrable; these were present, however, in lytically infected BHK-21 cells. The failure to detect membrane antigens on the chronically infected cells supports the view that rubella virus is probably passed as a cytoplasmic entity at cell division and is released into the fluid phase of the culture supernatant through internal or surface cellular membranes at such a low rate that the technique of immunofluorescence is not sensitive enough to detect it.

Rubella antigens were gradually lost from the cytoplasm of LLC-MK_2 cells. After the initial infection 80% of the cells stained positively with hyperimmune rubella antiserum, but after one year only 3% showed cytoplasmic antigen. The reasons why rubella persists in this small fraction of cells is unknown. Chronically rubella-infected cells produce large amounts of interferon but this does not appear essential for persistence (Stanwick and Hallum, 1974). There are two other mechanisms of viral persistence in cell culture which might apply to rubella: (a) integration of a DNA copy of rubella RNA into the DNA of the

cell chromosomes by means of the reverse transcriptase (RNA-dependent-DNA-polymerase) of a co-infecting retrovirus, and (b) persistence mediated through intracellular interference by defective interfering (DI) particles as described for vesicular stomatitis virus (VSV)(Huang, 1977).

The results of Sato et al. (1976, 1977) suggest that rubella virus might be integrated in the form of a DNA copy, making the cultured cells resistant to further superinfection. These workers claimed to find reverse transcriptase activity associated with the virions of a temperature sensitive mutant of rubella virus (pi variant) but not with those of a wild strain (MM-33). The claims recall the findings with a carrier culture infected with a Newcastle disease virus temperature sensitive mutant (Furman and Hallum, 1973). So far it is not clear whether all the necessary criteria have been met to confidently identify the enzyme as reverse transcriptase rather than a DNA-dependent DNA polymerase primed by RNA; also whether contamination with retroviruses was fully avoided. Some preliminary work in our laboratory (Norval and McLeod, pers. comm.) indicates that DNA extracted from rubella infected LLC-MK_2 cells is not infectious for RK_{13} cells. Thus, it did not produce rubella virus cytopathic effect in a transfection experiment; the DNA transfected RK_{13} cells were also negative for rubella antigens by indirect immunofluorescence. However, the extracted RNA from the LLC-MK_2 cells was infectious (Norval and Penny, pers. comm.). However, these studies were done with the DNA and RNA extracted from cells

chronically infected with a wild strain of rubella ('Thomas') and with the vaccine strain HPV-77 (Norval and McLeod, pers. comm.). It could be argued that they were not comparable with the experiments of Sato (1976, 1977) who used a ts mutant. So integration of DNA copy of rubella RNA viral genome remains a theoretical possibility but it is not likely to be the explanation in our persistently infected LLC-MK_2 cells.

In LLC-MK_2 cells persistently infected with rubella it appears that the rubella virus is retained in a form of infectious cytoplasmic RNA; the molecular mechanisms might be similar to those described for VSV. Defective-interfering (DI) particles in VSV persistent infection are antigenically indistinguishable from standard virus, but contain one third of the normal RNA genome. Interference is mediated intracellularly by competition at the level of genome replication, is strain specific, independent of interferon and is affected by host functions acting on nucleic acid replication (Huang, 1977). The VSV persistent infection depends on the slow release of infectious virions (Huang, 1973). The rubella persistently infected cells have not been studied in this light and so the importance of DI particles has to be proven experimentally.

Whatever the mechanism of persistence of rubella virus infection in LLC-MK_2 cells, the level of infection was a reasonable model for a hypothetical rubella infection within the rheumatoid joint and justified the use of these chronically infected cells in the developmental studies for the immunofluorescence and solid phase radioimmunoassay techniques.

Indirect immunofluorescence has been applied successfully to detect numerous viral antigens and it is widely used for the detection of cytoplasmic rubella antigens (Haire and Hadden, 1972a) as well as the detection of rubella IgM in patients' sera (Haire and Hadden, 1972b). When applied to the study of the rubella chronically infected LLC-MK_2 cells and lytically infected BHK-21 cells, cytoplasmic antigens could be clearly detected in both instances. The formol-saline fixation method proved an easy and convenient method for the detection of rubella membrane antigens in the lytically infected BHK-21 cells. The failure to demonstrate membrane antigens on LLC-MK_2 cells by immunofluorescence and their detection by radioimmunoassay is intriguing and poses the question of the relative sensitivity of the two methods.

Hayashi et al. (1973-1974) compared immunofluorescence with direct radioimmunoassay. They were able by radioimmunoassay to demonstrate viral antigen in various virus-cell systems, especially in the Herpes simplex infected cells, 1 - 2 hours after the absorption of infectious virions, at a time when the immunofluorescence was still negative for both membrane and cytoplasmic antigens. Unfortunately, the serum dilutions used, particularly for the immunofluorescence tests, were not mentioned in this communication.

Other studies (Hayashi et al., 1973; Forghani et al., 1975) have used indirect radioimmunoassay as a more sensitive method than direct radioimmunoassay. It was shown that the indirect

radioimmunoassay was more sensitive than immunofluorescence, at least for the detection of viral antibodies, because a 1:128,000 dilution of anti-Herpes simplex serum still bound three times more ^{125}I-labelled anti-IgG to the infected than to the uninfected cells (Hayashi et al., 1973). High dilutions of viral antibodies giving a substantial binding ratio were also observed with vaccinia infected cells (1:8,000). Such high dilutions of viral antibody were not positive by immunofluorescence although in these systems the relative sensitivities of the indirect radioimmunoassay and immunofluorescence for the detection of viral antigens were not tested.

In the present work, during the course of calibration of the indirect radioimmunoassay (RIA) for rubella, attempts were made to establish the titration end-point of the rabbit rubella antiserum and to find the optimal serum dilution, i.e., the dilution at which the binding ratio with rubella infected cells was the highest. Furthermore, the sensitivity of RIA was compared with immunofluorescence for the detection of rubella antigens. The HAI titre of the rubella antiserum was about 1:3,000. In the radioimmunoassay, at the dilution of 1:300 of anti-rubella serum, the rubella-infected cells bound 4.9 times more ^{125}I-labelled anti-rabbit IgG than the uninfected cells; this was the highest binding ratio obtained in this system. The binding ratio was only twice as high at the dilution 1:1,000. The immunofluorescence titre of the same rubella serum was 1:80 - thus confirming the claim (Hayashi et al., 1973), that the RIA is the more sensitive

of the two methods for the detection of viral antibodies. However, the detection of rubella antigen in lytically infected BHK-21 cells by RIA parallelled the immunofluorescence technique. It is calculated that the RIA was sufficiently sensitive to detect approximately one infected cell in 10,000 uninfected.

The results with RIA depended on numerous absorptions of the anti-rubella serum with uninfected control cells, hamster and human liver powders. The absorptions increased the differences between infected and uninfected cells (binding ratio) 2 - 3 times. On the other hand, variations in the amount of ^{125}I-labelled anti-rabbit immunoglobulin in the system did not influence the binding ratios. These observations agree with those of Forghani et al.,(1975).

RIA results based upon counting isotope emissions are more quantitative and less subjective than those based upon the microscopic reading of degrees of immunofluorescence. In general, it appeared to be a good technique to apply to the problem of detection of rubella antigens in synovial cells.

Although the model systems of chronically infected LLC-MK_2 cells and lytically infected BHK-21 cells gave clear cut positive results for rubella using IF or RIA with rabbit anti-rubella sera, the results with RA synovial fibroblasts and membrane sections by the same methods were negative.

This contrast with the observations of Patterson, Howard and Deinhardt (1973) described in the Introduction and since reiterated in a short publication (Patterson et al., 1977) that

appeared after the completion of the work described in this thesis; the discrepancy requires some exploration.

Two possible explanations come to mind. First, the Chicago workers used ^{51}Cr release from labelled fibroblasts exposed to rabbit complement and rubella antiserum. This technique might conceivably measure rubella antigens not detected by IF or RIA, either because of the greater sensitivity of the method, or because of a qualitative difference in antigen detection. Second, we used rabbit antisera, whereas the Chicago workers used a commercially available rubella antiserum (Microbiological Associates) raised in a rhesus monkey. Such an antiserum might be expected to contain (cross-reactive) antibody against components of the cultures used to grow the virus, as well as against viral antigens. The human sera used might contain antibody against rubella virus antigens not 'recognised' by the rabbit.

Current work in our laboratory is directed to checking some of these potential explanations. Correspondence with the Chicago workers and an exchange of reagents, revealed that the ^{51}Cr technique with the rubella antisera (human convalescent, rhesus, Edinburgh rabbit) is less sensitive than IF or RIA on infected LLC-MK_2 cells, although the presence of the virus in the line was demonstrated by interference with Coxsackie A_9. A particular strain of RA synovial fibroblast (S69) that had been found to give reproducible ^{51}Cr release with the rhesus rubella antiserum, and human antisera, together with a negative, normal human fibroblast (S77) were further investigated for the specificity

of the reaction. It was found that adsorption of reactive antisera with rubella virus infected cells did not remove the antibody reacting with S69 (Peterson, D., 1978: personal communication). It therefore appears that there is a second antigen-antibody system involved other than that of the rubella virus.

Tests in Edinburgh (Neill, W.A., 1978: personal communication) have confirmed the insensitivity of the ^{51}Cr technique for detection of rubella coded antigens when compared with IF (and by inference, given the same reagents, with RIA). Rubella infected LLC-MK_2 cells showing 5 - 10% positive immunofluorescence showed no ^{51}Cr release in the presence of rubella antisera and complement. Furthermore, the S69 strain of synovial fibroblasts, in our hands, is negative in a ^{51}Cr test with the rabbit and the rhesus antisera and also with a pool of human convalescent phase rubella antisera; it is also negative by IF (Neill, W.A., 1978: personal communication) and by the radiolabelled uridine incorporation method (although mycoplasmas were detected in the cells by this test and by culture).

Because of the failure to detect viral and other neoantigens on fibroblasts, the investigation for viral antigens has been extended to the vascular endothelium of the synovium, to other cells such as lymphocytes, macrophages or the chondrocytes of the cartilage.

Norton and Ziff (1966) and Hirohata and Kobayashi (1964) found that plasma cells and lymphocytes were the most numerous cells in the densely infiltrated areas of the subsynovium and

were arranged in three areas: (a) the lymphocyte rich areas; as much as 95% of the cells were of this type, (b) in the plasma cell rich areas in which the average plasma cell count was 67%, and (c) the transitional areas which are of particular importance because of the distribution of cell types and the importance of the inter-relationship between cell types. In these areas, lymphocytes and plasma cells were present at a ratio of approximately 2:1. Furthermore, blast cells and macrophages were present in much larger numbers than in the lymphocyte-rich zones or in any other location in the subsynovium. Ziff (1974) concluded that both T and B lymphocytes leave the synovial blood vessels and congregate initially around them to form the lymphocytic infiltrates. These areas would then be the 'lymphocyte-rich' collections. Presumably, as these cells come into contact with the (hypothetical) antigen in the synovial membrane, both types of lymphocytes would be expected to undergo blast transformation. The cells would ultimately differentiate into antibody secreting plasma cells and form the 'plasma cell rich' collections. The transitional areas, with the mixture of lymphocytes and other cells, may represent a cell-cell interaction area where lymphokines could be released and macrophages aggregate. This interpretation of the immunohistopathological findings might suggest the transitional area is the place to look for (presumptive) viral antigens. Rubella virus antigens were sought for in the transitional plasma cell and small lymphocyte area using sections of rheumatoid and osteoarthritic membranes. The immunoperoxidase

technique was used which was standardised by the detection of antigens in the liver of an infant with rubella syndrome. The results were negative.

The deposition of immune complexes in articular cartilage of RA patients is striking when compared with other arthritic conditions or osteoarthroses (Cooke et al., 1975a; 1975b). A relationship between immune complexes in RA articular and collagenous tissues, and synovial Ig synthesis, complement activation, and inflammation is discussed elsewhere (Cooke et al., 1975a; 1975b). As it is possible that the immune complexes in cartilage represent binding of antibody to a viral antigen in chondrocytes, a search for localisation/persistence of an extrinsic agent should include articular cartilage. The RA articular cartilagenous tissues in the present investigation were negative for rubella virus antigens. This finding would cover both (a) the persistent growth of rubella virus in the chondrocytes, similar to that observed in chondrocyte culture and cartilages of congenitally infected rabbits (Smith et al., 1973; London et al., 1970), and (b) the deposition or binding of immune complexes of rubella virus and antibody in the surface layers of cartilage.

Other cells investigated for the presence of rubella antigens by IF were the isolated synovial fluid lymphocytes. Rubella virus-infected lymphocytes have been shown to have a decreased response to phytohaemagglutinin (PHA) stimulation (McMorrow et al., 1974; Vesikari and Buimovici-Klein, 1975). Decreased responsive-

ness to PHA and other mitogens has been observed in RA synovial lymphocytes (Panayi, 1973; Stratton, 1972). However, my (limited) observations on synovial lymphocytes do not indicate the presence of rubella virus coded antigens or a persisting infection of the synovial lymphocytes. As has been already mentioned, rubella antigens were not found on synovial membrane lymphocytes.

There is, therefore, little evidence that a persistent rubella virus infection is the underlying stimulus for rheumatoid arthritis.

Rheumatoid polyarthritis after rubella

Despite the (continuing) failure to demonstrate rubella virus-coded antigens on a significant proportion of RA synovial fibroblasts, or on other synovial structural cells or lymphocytes, and to demonstrate increased levels of rubella antibody in the peripheral blood or in synovial membrane eluates, or immunoglobulin synthesized *in vitro* by synovial membrane fragments from RA patients, the sporadic occurrence of cases such as Mrs A. prevent the outright rejection of rubella virus as a causative agent in RA and requires an explanation.

Mrs A's initial illness certainly resembled rubella in clinical terms. Her subsequent polyarthritis was clearly similar to mild rheumatoid arthritis and tests for rheumatoid factor were positive. However, despite the synovitis and the effusions, there were no erosions of bone and cartilage and a complete

recovery took place without deformity.

Rubella virus antigen (or virus) was not demonstrated in the joints although the pattern of IgM rubella antibody response suggested a persistent infection with rubella virus. One possible explanation for the phenomenon may be that the rubella virus infection was at an extra-articular site - e.g., throat, cervical lymph nodes, other lymphoid tissue - and that the arthritis may, in fact, have been of the reactive variety rather than RA and mediated by lodgement of circulating immune complexes in the joints (intracellular inclusions of IgG and IgM were seen in joint phagocytes). The pathogenesis of the condition would then be in line with the polyarthritis of Hepatitis B, but more prolonged.

The throat, circulating lymphocytes and other extra-articular tissue should have been examined for rubella virus, particularly at the time of the "upper respiratory infection" in December 1970 when her condition relapsed and the polyarthritis became severe. It would also have been of interest to know whether the synovial fluid contained IgG RF of the self-associating type found in RA; the RF tests done were on peripheral blood and detected IgM RF, which may be stimulated by chronic infections as well as by the rheumatoid process.

Immunoglobulins synthesized or bound by rheumatoid synovial membranes

The apparent commitment of the RA synovial lymphoid infiltrate to produce antibody to an (unidentified) antigen (Herman et al., 1971) and the ability of the membrane to synthesize various classes of immunoglobulin in vitro (Smiley, Sachs and Ziff, 1968) have been outlined in the Introduction. Examination, in the present investigation, of synovial eluates, or Ig produced by synovial lymphocytes in vitro has not revealed substantial amounts of antibody to adenovirus, measles, rubella or mammalian retroviruses.

Since the study was concluded, other workers have also provided evidence that the locally-produced immunoglobulins do not contain anti-viral antibodies. Standford (1974) did not find an increased level of viral antibodies in synovial fluid when compared with that in peripheral blood. Cryoprecipitates from rheumatoid synovial fluids do not contain more viral antibodies than those from the peripheral blood of the same patients (Cremer et al., 1974). Also acid or other eluates from RA synovial membrane do not reveal striking amounts of viral antibodies (briefly mentioned in Zvaifler, 1973).

This study is however the only one that used the immunoglobulins produced in vitro by the synovium membrane and labelled with ^{14}C-amino acids for viral studies. Direct analysis of radiolabelled immunoglobulins offers several theoretical advantages: (a) it distinguishes locally-produced immunoglobulins from immunoglobulins or immune complexes which could be synthesized

at an extra-articular site and be selectively trapped in joints, and it also distinguishes them from cross-reacting antibody with synovial membrane; (b) unlike examination of eluates, it does not require high concentrations of immunoglobulins in the synovial membrane and fluid which might not be maintained if the locally-produced immunoglobulins form immune complexes with 'the local antigen' and are efficiently removed by phagocytic cells.

The model system used to calibrate the method was the incorporation of ^{14}C amino acid into immunoglobulins by spleen fragments from a rubella inoculated rabbit. Some of the results obtained with various serological tests aimed at detecting the ^{14}C-antirubella were however rather unexpected. The tests used were (a) radioimmunodiffusion, (b) autoradiography, (c) sucrose gradient analysis.

Radioimmunodiffusion technique, i.e., the formation of a precipitin line between antigen and unlabelled specific antibody, followed by diffusion of the unknown radiolabelled antibody would have been simple to use and has been applied to other systems (Herman, Wiltse and Dennis, 1973). However, concentrated rubella antigen and either the rabbit hyperimmune serum or human rubella convalescent antibodies did not form precipitin lines, with the exception of a rubella antiserum (R1/HPV 77/4-7.1.75) which cross-reacted with foetal calf serum. The other rabbit rubella antiserum, carefully prepared in a homologous system (rubella virus grown in RK_{13} cells in rabbit serum) did not react with calf serum and did not precipitate with concentrated rubella

antigen. This was unexpected in the light of reports in the literature.(Le Bouvier, 1969).

Several reasons for this result can be envisaged - insufficiently concentrated rubella antigen, lack of precipitating antibody in the sera, or artefacts in the published reports due to a failure to recognise the presence of contaminating antigen-antibody systems.

The concentration of rubella antigen paralleled the titre of rubella virus in the fluid phase of the culture and fell in the same range indicated by Le Bouvier (1969). Le Bouvier used as antigen, concentrates of medium from cultures of the PS (porcine stable) line of pig kidney cells chronically infected with rubella virus: 10^5 focus forming units of virus/ml were present. In the present study the fluid phase of a lytically infected culture of Vero cells was used that also had a virus titre of 10^5/ml. The Vero cells produced high titres of rubella virus (Liebhaber, Pajot and Riordan, 1969), substantially higher than those in the chronically infected HPV-77 LLC-MK_2 cell culture. The concentration of antigen was therefore likely to be adequate for precipitation to occur.

An absence of precipitating antibody in the hyperimmune rabbit serum might have been the reason. Although the rabbit is known to produce good precipitating antibody, later in the response non precipitating antibody can take over. However, a likely reason is that cross-reactions with contaminating antigens, other than rubella were not fully considered by Le Bouvier (1969). The hyperimmune baboon rubella antiserum he used along with the

partially purified rubella virus would be expected to react with calf serum and pig cellular antigens. It is significant that other workers have failed to repeat Le Bouvier's observation of two rubella precipitating antigens (Dr H. Gould - pers. comm.).

Two other novel approaches were used in the present work for the analysis of radiolabelled immunoglobulins. The autoradiography method was an extension of the direct immunofluorescence technique on rubella infected LLC-MK_2 cells which were positive by indirect immunofluorescence for cytoplasmic antigens. Stripping film emulsion autoradiography has been used successfully for the detection of Herpes and vaccinia virus antigens. Theoretically, it should have been possible to layer specific ^{14}C-labelled rubella antibody over the infected cells and follow the distribution of the sensitised emulsion grains in the cytoplasm of the infected cells. The most likely explanation of the failure to detect the labelled rubella antibodies was that the concentration of radiolabel was not sufficient to sensitise the film emulsion during the exposure time.

The third approach to the analysis of radiolabelled immunoglobulins was dependent on the formation of an antibody-virus complex composed of ^{3}H-uridine labelled virus and ^{14}C-labelled immunoglobulins with different sedimenting density for the complex and virus. In the control system ^{14}C immunoglobulins produced in the rubella infected rabbit spleen system formed immune complexes with rubella that was easily detectable by the shift in density from 1.18g/cm^3 for rubella alone to 1.24-1.25g/cm^3

for the immune complexes. There was also detectable increase in the ^{14}C counts in the region of the virus-antibody complex.

Sucrose gradient analysis offered a quantitative approach to the study of specific immunoglobulins in that the proportion of the virus specific antibody to the total protein synthesized could be determined, and secondly, a titration of the virus specific antibody could be done by diluting the starting material. However, in this study of rheumatoid synovial membrane immunoglobulins no attempt was made to quantitate the results. It was concluded that specific viral antibody was present in the membrane immunoglobulins when two criteria were fulfilled simultaneously: (a) a shift in the density of the viral peak, and (b) when the immune complex peak contained more ^{14}C-counts at the density appropriate for immune complexes than RA membrane supernatants spun on their own. According to these criteria all the synovial membrane preparations, with one exception, were negative for viral antibodies. Membrane 720222, the exception, gave a positive result with rubella virus. This observation was interesting but it is difficult to assess its significance in the context that all other attempts to detect rubella antigens in the rheumatoid synovial membranes were negative. Also the numerous acid eluates prepared from rheumatoid synovial membranes showed no haemagglutination-inhibiting rubella and measles antibodies, thus making this isolated result less significant. Generally the conclusion must be that the locally produced immunoglobulins are not viral antibodies against the candidates tested.

Retroviruses and Rheumatoid Arthritis

(a) Antibodies reactive with primate retrovirus antigens

Antibodies to endogenous retrovirus envelope and core antigens can be detected in mice (reviewed Ihle and Hanna, 1977). This suggests that mice are not immunologically tolerant to a spontaneously activated endogenous murine leukaemia virus (MuLV) and that serological investigation can be used to detect activated endogenous virus.

Several groups of workers have examined normal human or patients' sera for antibodies to primate retrovirus antigens using different methods of detection and with differing results.

Radioimmunoprecipitation (RIP) has been the most popular method. Stephenson and Aaronson (1976) tested 100 sera from cancer patients and found no antibodies to SSAV or MuLV (antigens p 30 and gp 70). Sera from 35 lupus erythematosus patients were included as controls and were also negative. Antibodies were not detected to GaLV (gibbon ape type-C leukaemia virus) although it was established that cancer patients are capable of responding to immunisation with formalin-inactivated Rauscher leukaemia virus (p 30 and gp 70 antigens) (Charman et al., 1975).

On the other hand, Snyder, Pincus and Fleissner (1976) using two different sources of purified SSAV antigens (grown in KNKR and NC-37 cells), detected reactivity in normal human sera by RIP. The activity was associated with IgG, and with Fab 2 fragments in particular. The antigens that reacted were clearly present in larger amounts on SSAV producing cells, as compared

to non-producing cells. Nevertheless these workers wisely considered the possibility that non viral antigens might be responsible for the reactions. Expression of Forssman-type haptens on the surface of retrovirus-transformed mammalian cells (Burger, 1971) was ruled out, as absorption of the positive human sera with SRBC did not alter the radioimmunoprecipitation curves. The results of quantitative absorption tests with tissue culture cells argued against cellular antigens being involved. Some inhibition was however achieved when human sera were absorbed with foetal calf serum (FCS). This might be a real problem in all studies of anti-retrovirus antibodies as normal human sera show reactivity with an as yet unknown component of FCS that can absorb on to infected cells from tissue culture fluid (Hirshaut et al., 1974). Snyder and his colleagues however argued that the FCS component was not fully responsible for results obtained because (i) human sera precipitated greater amounts of virus than rabbit anti-FCS; (ii) three other viruses grown in cells in the presence of 10% FCS did not react with human sera; (iii) human sera precipitated only tiny amounts of ^{125}I-labelled FCS. The matter is unresolved but this work has at least shown the care that must be taken in similar studies. The titres of retrovirus 'antibody' in the normal human sera were usually low, with a maximum titre of 320 by RIP as compared with the positive control animal sera in Stephenson and Aaronson's (1976) studies that had titres of 800-12,000. Kurth et al. (1977) also studied 'antibodies' in normal human sera that reacted with primate retrovirus

antigens in RIP. Of 39 sera tested 100% reacted with baboon endogenous virus (BEV) and 49% reacted with simian-sarcoma associated virus (SSAV). The titres were low, in the region of 80-160. Again, the possible artefacts arising from Forssman antigen were excluded by absorbing the sera with SRBC. However the effect of the FCS component absorbed from the tissue culture fluid was not controlled; nor were there controls for the antigens of the cells used to grow the virus from which the specific antigens were extracted and labelled.

Other serological methods, such as the inhibition of reverse transcriptase (RT) activity of primate oncornaviruses by 7S immunoglobulins were negative for normal sera whereas immunoglobulins from two of eight leukaemia patients significantly inhibited the incorporation of ^{3}H-TTP by the RT of HEL-12 virus (Prochownik and Kirstein, 1976). The sample studied was small, but indicated that this anti-enzyme activity is absent in normal human sera. Another approach was used by Aoki et al. (1976). This is an electron microscope technique with antibody labelling the virus envelope and followed by ferritin conjugated goat IgG with anti-human IgG activity. Labelling of whole virus envelope was considered specific. 78% of normal human sera showed a positive reaction. The evidence for specificity was slender; some attempts were made to absorb the human sera with FCS but there was no attempt to absorb with uninfected cells or with SRBC to exclude other cross-reactivities.

To summarise the studies so far performed it seems that

normal human sera may possess low levels of specific reactivity with retrovirus antigens. If retrovirus activation or infection is involved in RA then it might be expected that more substantial levels of antibody would be found in patients, when compared with controls. The method used in the present study for detection of antibodies to primate retrovirus antigens differed from those just reviewed in that it used solid phase radioimmunoassay (RIA) rather than RIP. RIA with infected cells as the solid phase has several advantages: (i) there is no need for lengthy purification of the viral antigens; (ii) all viral antigens are present in the acetone fixed infected cells (core antigens, envelope antigens, reverse transcriptase); (iii) an additional control can be set up for all dilutions of human sera; viz. the counts bound to the uninfected cells can be subtracted from counts bound to the virally infected cells, thus binding to cellular antigens and foetal calf serum absorbed from tissue cultures will be excluded from each assay. Although it is not possible to compare the sensitivity of this method to that of previous work, it seems that it might be of a similar order.

As shown in the Results, positive rabbit anti-sera to SSAV and RD-114 gave titres of 10,000 and 3,000 respectively. Dilutions of human sera of 100, 300, 1,000 were chosen on the basis that if retroviruses were involved in 'autoimmune' diseases there should be a substantial difference in binding to infected compared to uninfected cells. The range of antibody titres in the immune response to endogenous virus in mice is 0 - 5120 by RIP (Ihle

and Hanna, 1977) and 0 - 320 in normal human sera (Snyder, Pincus and Fleissner, 1976). Human sera were absorbed with SRBC to remove Forssman antigen cross-reactivity, thus making the set of controls complete.

There was no difference in binding in the assay between the three groups of subjects tested, i.e. RA, SLE and non-RA controls. These negative results are valuable as a contribution to the vexed question of whether human beings have antibody to retrovirus, quite apart from their interest in relation to rheumatoid arthritis. They also agree with unpublished observations from other groups. Snyder and his colleagues did not find any difference in the distribution of low levels of the presumptive anti-retrovirus antibodies in normal controls and in patients with autoimmune diseases (Snyder, Pincus and Fleissner, to be published).

The study here was extended further by absorption of RF. RF is known to interfere with complement fixation tests and could conceivably mask the second step of the RIA by blocking the binding of ^{125}I-anti-human IgG. The removal of rheumatoid factor did not alter the relative distribution of binding values between RA and other diseases although there was some change in absolute values.

These results do not support the notion that activation of endogenous human retrovirus, or infection with an exogenous virus, is the underlying stimulus in rheumatoid arthritis. This study also throws doubt on the involvement of this group of

viruses in systemic lupus erythematosus.

It could be objected that the approach used here would not detect antiviral antibodies if these were bound in immune complexes. A mechanism of this type has been shown in animal studies, in particular previous work on the AKR mice, a strain characterised by continuous high levels of virus expression, with deposition of immune complexes in the kidneys (Batzing, Yurconic and Hanna, 1974) but absence of free antibody in the serum. In other strains (NIH3 Swiss, SWR/J), in contrast to the AKR strain, the lack of demonstrable antibodies is correlated with the absence of endogenous virus expression in these animals.

In the 'autoimmune' complex of diseases of the NZB and (NZB x NZW)F_1 mice, which has been associated with the expression of endogenous retrovirus, acid eluates from kidney show selective concentration of retrovirus soluble antigen during the early (2 weeks) life of the animal; free antibody in serum could be detected only later (14 weeks) by indirect immunofluorescence (Mellors et al., 1971).

Although it seems likely that in our study the absence of retrovirus antibodies in the human sera reflects the absence of the endogenous virus expression (see next section of the discussion), an extension of the work to cover removal of circulating antibody in immune complex deposits is now in progress. Eluates from rheumatoid synovial membranes will be tested by immunofluorescence, or RIA, for the presence of antibodies, thus completing this aspect of the investigation.

(b) Retrovirus antigens on RA lymphocyte membranes

The interactions between lymphocytes and retroviruses are complex and poorly understood. From a murine model of the immunological activation of endogenous murine retroviruses, the activated viruses are predominantly associated with T-lymphocytes in vivo (Hirsch, 1976). Other observations suggest that lymphocytes at certain stages of differentiation may be highly susceptible to activation of endogenous virus (Obata et al., 1975). From studies, in vitro, on the mixed lymphocyte reaction (MLR), the activated viruses detected were observed by electron microscopy to bud exclusively from lymphoblasts (Andre-Schwartz et al., 1973).

At present the evidence for the presence of endogenous retrovirus in normal human cells is still only circumstantial. The intensive search has been sustained by numerous laboratories as similar isolations in other animal species have been achieved (for review see Aaronson and Stephenson, 1976). Stephenson and Aaronson (1976) studied 200 cell cultures of human origin and failed to detect spontaneous viruses of this group or to induce them chemically. However fusion with cell lines of other primate species might be a necessary step in order to detect a xenotrophic endogenous human virus. This contrasts with a single report of an isolation of a retrovirus from normal human fibroblast culture (Panem et al., 1975). There is also agreement among several laboratories regarding the absence from human cellular DNA of viral nucleic acid sequences homologous with the

SSAV/gibbon ape group and the baboon endogenous virus (Scolnick et al., 1974; Benveniste et al., 1974; Wong-Staal, Gallo and Gillespie, 1976); thus suggesting that normal humans do not carry these sequences in their genome.

The majority of normal human tissues, including lymphocytes, appear to lack detectable antigenic cross-reactivity with SSAV p30 (Stephenson and Aaronson, 1976). This result contrasts with previous reports on crude extracts of human tissues (Strand and August, 1974; Sherr and Todaro, 1975) although the later work (Stephenson and Aaronson, 1976) showed that exacting purification steps are required to remove non-specific reactivity against human tissues.

However, there are reports of (possible) exogenous or infectious human retroviruses isolated from lymphoid cells of leukaemic patients. Repeated isolations of virus were made from the blood cells of one patient with acute myelogenous leukaemia (AML) by Gallagher and Gallo (1975) and later a virus with apparently the same characteristics was isolated from the same patient's bone-marrow cells (Gallagher et al., 1975). These have been the best characterised isolates of a human C-type virus (HL23V).

Immunological and biochemical characterisation of the viruses isolated from human AML cells showed a relationship to the exogenous primate viruses of SSAV/gibbon ape and the endogenous group of RD-114/baboon (Teich et al., 1975; Okabe et al., 1976). This was confirmed by hybridisation studies (Chan et al., 1976).

Both groups of workers suggested that a mixture of two viruses were present. All isolates replicated well in human cells (Teich et al., 1975). Repeated isolation from human leukaemic bone marrow cells of virus related to SSAV has been reported by Nooter et al. (1975). Kaplan et al. (1977) recently described the spontaneous release of a retrovirus from a human histiocytic/lymphoma cell line. This proved to be related to SSAV and RD-114 viruses as tested by inhibition of reverse transcriptase(RT) activity. The isolate (HEL-12) from the normal human fibroblast culture mentioned earlier (Panem et al., 1975) is likely to belong to this group of viruses and again shows relatedness to SSAV and RD-114-like antigenic components, measured by immunofluorescence and by inhibition of RT activity (Panem et al., 1977) and replicates well in human cells. The trend towards the isolation of viruses related to SSAV or RD-114 is impressive. The isolations are, however, infrequent as observed by Stephenson and Aaronson (1976) who systematically followed up 32 tumour cell lines without success.

The possible involvement of viruses related to SSAV and RD-114/BEV groups in human malignancy has been shown by the detection of proviral sequences of baboon endogenous type-C RNA virus in DNA from human leukaemic tissues (Wong-Staal, Gillespie and Gallo, 1976) but others have failed to detect sequences related to SSAV (Tavitian, A. - in Wong-Staal, Gillespie and Gallo, 1976). Reverse transcriptase activity seems to be readily detectable in human acute leukaemic cells (Sarngadharan

et al., 1972; Baxt, Hehlmann and Spiegleman, 1972) and is apparently clearly distinguishable from the DNA polymerases of normal human lymphocytes, thus indicating that retroviruses may be associated with leukaemia.

The association of retroviruses with autoimmune diseases might be credible if an endogenous xenotrophic human virus rather than the infectiously transmitted virus is implicated (Teich and Weiss, 1977). The association of a murine retrovirus with the 'autoimmune' haemolytic anaemia and glomerulonephritis in New Zealand mice (Levy, 1974) and the presence of retrovirus genetic information in latent or partially expressed states in so many vertebrate species, makes this suggestion attractive. Xenotrophic endogenous viruses, genetically transmitted, are not infectious for their own species in culture (Levy, 1974) and therefore would not be detected as a persisting infection in normal human cell cultures.

The serological study of rheumatoid synovial and peripheral blood lymphocytes described in the Results was initiated with the knowledge of the cross-reactivity of the known human C-type virus isolated with the SSAV/gibbon ape primate group of infectious virus and the endogenous group of RD-114/baboon. Initial results of the study using goat sera supplied by Dr J. Gruber, NCI, Bethesda were exciting. The immunofluorescence with anti-SSAV (TE) and anti-RD-114 (TE) goat sera gave a series of positive results with rheumatoid synovial lymphocytes, while other goat sera were negative (anti-murine leukaemia virus, anti-

feline leukaemia viral proteins p12 and p27 and anti-Mason Pfizer Mammary tumour virus proteins p12 and p27). Superficially this fitted the pattern of cross-reactivity observed with the recently isolated human C-type virus. The excitement was short-lived when further experiments revealed that these sera also stained normal human lymphocytes at lower intensities, agglutinated human red blood cells and cross-reacted with human IgG coated sheep blood cells (data not shown) thus indicating that the main part of the immunofluorescence observed was due to the cross-reaction with 'normal' human antigens.

Because of these non-specific serological cross reactions one must question the observations of Panem et al. (1977) on the relatedness of HEL-12 virus isolate to SSAV and RD-114, as these researchers used sera from the same source (Dr J. Gruber, NCI, Bethesda). These sera would also cross-react with cellular antigens and between viruses grown in homologous cell lines.

That cellular antigens are specifically incorporated into the viral particle of Friend leukaemia virus was shown by the association of H-2 antigens with the core of the virions (Bubbers, Chen and Lilly, 1977) and not into the viral envelope as one might predict from the mode of viral maturation (Bubbers and Lilly, 1977).

In this circumstance even purification of viral antigens and the separation of viral proteins by gel-chromatography would probably not remove the 'cellular' contribution, so subsequent immunisation will yield antibodies that cross react with cell culture antigens and also serum absorbed from the culture fluid.

The best way to overcome this problem is to immunise an animal with virus grown in cells and serum of the same species - e.g. a rabbit with viruses grown in rabbit cells and normal rabbit serum. This approach was used in the production of rabbit anti-RD-114 serum. This reagent was then used as a tool in the present immunofluorescence, radioimmunoassay and complement dependent cytotoxicity studies to investigate whether retrovirus antigens were expressed on rheumatoid synovial lymphocytes as this could be a cause of the 'disordered immunoregulation' observed in rheumatoid patients. All tests employed were negative, except for one case of juvenile rheumatoid arthritis, whose peripheral blood lymphocytes showed the presence of retrovirus antigens on the cell surface as measured by radioimmunoassay; the synovial fluid lymphocytes from the same patient were negative. The level of serological reactivity was comparable to that of RD-114 infected RD cells. This interesting observation could not be followed up quickly enough to include it in this thesis (the patient left for Germany) but more work is now in progress to repeat the observation and to attempt an isolation of retrovirus from this patient's peripheral blood lymphocytes.

Anti-SSAV (TE) sera produced in rabbits showed no specific viral antigens on the surface of rheumatoid synovial or peripheral blood lymphocytes. This antiserum was not produced using the homologous system as the attempts to grow SSAV virus in RK_{13} cells were not successful (data not shown). But unlike the goat serum from NCI, the cells used to produce the immunising viral antigens were at least of a different (rat kidney) species to the eventual test cell. The antiserum cross-reacted strongly

with rat cells and a low level of cross-reactivity with normal human cellular antigens was removed by absorption. The sera were specific for the viral antigens, i.e. the anti-RD-114 serum did not stain SSAV infected cells, consistent with observations by others (Panem et al., 1977).

An extension of this work pertinent to rheumatoid lymphocytes was also carried out by trypsinising the cells prior to the immunological tests. The work of Wangel and Klockars (1977) showed that immune complexes will block SRBC receptors on rheumatoid T-lymphocytes and these can be removed by trypsinisation. The SRBC receptors were allowed to regenerate by culturing the lymphocytes overnight in the absence of immune complexes. The same method of trypsinisation and regeneration of 'presumptive' viral antigens on lymphocyte cell membranes was followed. This did not however yield any positive results with specific viral antisera. The lymphocyte preparations also included cells from enzyme digested synovial membranes.

(c) Retrovirus antigens in synovial membrane cells or immune complexes

Retrovirus antigens were detected in the mesangium and along the glomerular capillary walls in human SLE kidney (Panem et al, 1976), the same location as described in the murine model of 'autoimmune' immune complex disease. An analogous location in the rheumatoid membrane would presumably be the vascular endothelial cells and the areas formed by the immunological

infiltrates (see section of rubella studies). Both frozen section and paraffin sections of synovial membranes were stained by immunofluorescence and immunoperoxidase. The IgG and IgM containing complexes could be easily identified in the sections when present, but no specific staining was detected when using anti-retrovirus sera, thus suggesting that these complexes did not contain retroviral antigens. Therefore it is unlikely that persistent viraemia or activation of human endogenous retrovirus is responsible for the formation of these immune complexes. No other cell type (synovial lining cells or macrophages) possessed viral antigens, therefore covering the possibility that a cell different to that of a lymphocyte could be expressing the viral antigens.

General Discussion

Is there cause for a virologist to continue the laborious investigations into the possible presence of viruses in rheumatoid arthritis? As a result of recent work and the study set out here, the chance of finding complete virions is rapidly diminishing. If persisting infection is thought to be an underlying continuous stimulus in RA the virus would have to be highly defective, expressing only few viral antigens. If this is the

case, then perhaps the candidate viruses chosen were 'wrong', or 'wrong' synovial cells were used in the search.

Rubella and retroviruses were the best choices in light of the knowledge available at the time. In the case of rubella virus most host-virus relations have been covered and all synovial membrane cell types were investigated with no positive results.

With retroviruses attention was concentrated on the detection of antibodies, in groups of patients with 'autoimmune' diseases, but without success. Other work carried out in our laboratory at Edinburgh included the labelling of lymphocytes with ^{3}H-uridine and ^{3}H-thymidine; even after stimulation with PHA the labelling did not reveal any virions production by these cells (Norval, to be published). Additionally, PEG fusion of RA synovial fluid lymphocytes with retrovirus-permissive cell lines was carried out and the resultant heterokaryons were examined by ^{3}H-uridine labelling, reverse transcriptase assay and by immunofluorescence for antigen; all were negative (Norval and Hart, to be published). The investigation of retrovirus antigens on the synovial lymphocytes described in this thesis explored the last remaining possibility of implicating retroviruses in RA; it was also negative.

Could other viral candidates be more suitable? At the present moment there is no indication of any specific viral involvement and the evidence for their possible role still remains only circumstantial.

However, there is considerable exciting new research which

one feels is going to become important in the possible viral aetiology of rheumatoid arthritis. The discovery by Zinkernagel and Doherty (1975) of the H-2 region histocompatability requirement in mice for cytotoxic killing of virally infected cells is now becoming a classic and has stimulated an enormous amount of thought and work. The advantage of this requirement to an animal has been questioned, but it seems that the diversity and the polymorphism of H-2 molecules is linked to enhanced immunological surveillance and presumably contributes to variation in the immunogenicity of the "altered self" (Doherty and Zinkernagel, 1975). The quantitative expression and synthesis of H-2 (HLA antigens in humans) antigens in virus infections has also been suggested as a link to the control of virus-induced neoplasia (Meruelo et al., 1978). Is it possible to link these findings to RA aetiology? There are some interesting points of association which, although still very questionable, one feels might be important enough to be investigated.

The question of what stimulates the production of self-associating IgG rheumatoid factors in RA remains unanswered. The classical suggestion of auto-sensitisation to IgG is still only explained by hypothetical models (Johnson, Watkins and Holborow, 1975). Another suggestion would be a mitogenic stimulation of self-reactive clones of B-cells by a virus (a mechanism which is likely to be important in EBV virus infection for the production of autoantibodies in vivo) or a persisting infection of these clones causing transformation of B-cells

already committed to produce auto-antibodies (the mechanism of antibodies production in EBV infected lymphoblastoid cell lines *in vitro*). This seems unlikely to be the mechanism of the autoantibody production in RA. The singular specificity i.e. IgG molecules with anti-human IgG Fc portion specificity and now even with anti-nuclear factor activity associated with the same $F(ab)_2$ fragments (Hannested and Johannessen, 1976) makes it improbable. The chance of hitting and stimulating the self-reactive clones of this dual specificity is extremely small when compared to the frequency with which it occurs in RA. Yet there is also some clonality of the production of the IgG rheumatoid factors in the rheumatoid synovial tissues, suggesting that a single B-blast cell is either planted in the synovium and proliferates locally or that the single B-lymphocyte is stimulated in the RA synovial membrane.

Other cells in the synovial membrane are the T-cells or null cells (Tannenbaum *et al.*, 1975). T-lymphocytes can increase synthesis and expression of H-2 antigens as a result of virus infection (Meruelo *et al.*, 1978). Associated with H-2 and HLA antigens is non covalently bound β_2 microglobulin (Peterson *et al.*, 1976; Callahan and Allison, 1978) whose production by lymphocytes can also be enhanced by virus infection (Meruelo *et al.*, 1978) or PHA stimulation (Bernier and Fanger, 1972).

Is it possible to link these observations? There is a striking homology between the β_2-microglobulin and human IgG especially the CH3 domain (Peterson *et al.*, 1972; Smithies and

Poulik, 1972). Could it be that there is a break point of immune tolerance when the quantitative expression of the histocompatibility antigens (and β_2-microglobulin) reaches a certain level under the influence of single or repeated viral infections of T-lymphocytes with consequent production of antibodies against the β_2-microglobulin. The antibodies against β_2-microglobulin, because of their affinity for C3 domain of the Fc portion of IgG molecule will have a tendency to self-associate within the plasma cell to form immune complexes. They will be biologically inactivated by the self-association and this in turn could lead to a break down of the antibody-feedback control mechanisms, resulting in chronic antibody production. Another mechanism of perpetuation of the inflammatory response could be the B-lymphocyte mitogenic activity of the anti-β_2 microglobulin molecules (Möller and Peterson, 1975; Ringden and Möller, 1975). The IgG immune complexes could in turn stimulate low affinity self-reactive clones to produce the classical IgM rheumatoid factor. It would be possible to test this suggestion by reacting F(ab)2 fragments from IgG RF and with specificity for human IgG (Fc) on mitogen stimulated T-lymphocytes or T-lymphocytes obtained from rheumatoid synovial membranes. The fragments should also preferentially react with β_2-microglobulin or autologous HLA complexes. Workers in the rheumatology field have looked for the HLA specificity of the lymphocytotoxins found in rheumatoid sera (Raum et al., 1977) without detecting any. These workers however could be dealing with a different system of cytotoxicity

mediated through immune complexes by Fc-Fc bridging, which has been suggested as an immunological effector mechanism, active only within the lymphoid system (Jondal, 1977). This view is supported by the increased reactivity of lymphocytotoxins at 4°C which seems to correlate with the formation of cryoprecipitates and is still of questionable value in vivo. Digestion of Fc-portion with pepsin prior the reaction should eliminate this mechanism.

The mechanism of initiation of RF production suggested here would be one where viral infection would be essential to initiate the events but would not have to become a persisting one to continue the events leading to definite or classic RA. Only recently some new experimental models are beginning to emerge of autoimmunity induction employing the injection of virus-modified cell membrane antigens into syngeneic mice (Eaton and Almquist, 1977). While the injection of uninfected lymphoma membranes or virus on its own did not stimulate production of auto-antibodies, the uninfectious NDV (Newcastle disease virus) virus-modified lymphoma membranes did. Antibodies to normal spleen, kidney and lung homogenates could be detected. Heterophile antibodies agglutinating rabbit immunoglobulin coated sheep red blood cells (RF like reactivity), also anti-DNA antibodies and anti-DNP antibodies appeared relatively early during the course of immunisation. The mechanisms behind the autoimmunity induced by the virus-modified membranes are intriguing and if these experiments stand up to the test of time

this will become an important area for a virologist to continue the search for the mechanisms of the possible viral aetiology of rheumatoid arthritis.

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ABBREVIATIONS

AML	Acute myelogenous leukaemia
BEV	Baboon endogenous virus
Con-A	Conconavalin A
FCS	Foetal calf serum
FeLV	Feline leukaemia virus
KNRK	Kirstein virus transformed normal rat kidney cell line
KW23	SSAV chronically infected KNRK cells
MuLV	Murine leukaemia virus
OA	Osteoarthroses
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline (pH 7.2; 0.01M)
PHA	Phytohaemagglutinin
PWM	Pokeweed mitogen
RA	Rheumatoid arthritis
RD	Human rhabdosarcoma cell line
RD-114 RD	RD-114 virus infected RD cells
RD-114 RK_{13}	RD-114 virus infected RK_{13} cells
RF	Rheumatoid factor
RIA	Solid phase radioimmunoassay
RIP	Radioimmunoprecipitation
RT	Reverse transcriptase enzyme
SFL	Synovial fluid lymphocytes

SFLTR	Trypsinized synovial fluid lymphocytes
SLE	Systemic lupus erythematosus
SM	Synovial membrane
SSAV	Simian sarcoma associated virus

APPENDIX I

Peroxidase - anti - peroxidase (PAP)

Peroxidase - anti-peroxidase PAP

(This is horseradish peroxidase antigen complexed with rabbit antiserum to horseradish peroxidase)

Technique:-

1) Take sections to ethanol.
2) Treat with fresh 1% hydrogen peroxide (30 vol) in methanol. 30 Min.
3) Transfer staining rack directly to tris-saline. 3 changes. 5 mins.
4) Block background tinus with normal swine serum 1:20 in tris buffer. 10-15 Mins.
5) Pour off and without washing introduce specific immunoglobulin a 1:100 dilution K & L or 1:50 IgA, G, or M, in Tris buffer greater dilutions may be required. 30 Min.
6) Wash off with squeezy and 3 dishes of tris-saline. 5 Min.
7) Drop on a 1:20 dilution of Swine anti rabbit serum specific to IgG in Tris. 30 Min.
8) Wash in tris-saline as before. 5 Min.
9) React with PAP 1:100 in Tris. 30 Min.
10) Wash in tris saline as before. 5 Min.
11) Wash as before (stage 6).
12) Stain with Diaminobenzidine 6 mgm in 10 ml Tris. add 2 drops 10 vol. H_2O_2 must be fresh (or one

drop 30 vol.) this solution should be colourless and is stainworthy for possibly 30 min. However use immediately. Staining time 4 min.

13) Wash in water, counterstain in haematoxylin 2 - 3 Min. Wash, differentiate if required, blue up.

14) Wash, dehydrate, clear and mount in HSR.

APPENDIX II

Publications from this work

Annals of the Rheumatic Diseases, 1977, **36**, 3–12

Rubella virus and rheumatoid arthritis

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SUMMARY A collection of synovial fibroblasts from 19 patients with rheumatoid arthritis (RA) and 12 patients with osteoarthrosis or other non-RA disease has been examined for rubella virus antigens by immunofluorescence and radioimmunoassay with negative results. Eluates of synovial membrane prepared under conditions likely to dissociate antigen-antibody complexes have shown no rubella antibody. A serological survey of RA patients and those with other forms of arthritis has shown no differences in the frequency or levels of rubella haemagglutination-inhibiting antibody. These results provide little support for various hypotheses that a persistent infection with rubella virus underlies or initiates the rheumatoid process.

The phenomenon of persistent infection with rubella virus is familiar from the congenital rubella syndrome and from some patients with a variety of subacute sclerosing panencephalitis (Townsend *et al.*, 1975; Weil *et al.*, 1975), and also from persistent infection of cultured cells *in vitro*. The tendency, taken with the observation of a self-limiting synovitis as an occasional complication of acute rubella, lends attraction to a hypothesis that a persistent infection of synovial cells with rubella virus might be the ultimate antigenic stimulus in juvenile (JRA) or adult rheumatoid arthritis (RA).

To test this hypothesis numerous workers have compared the frequency, or mean titre, of rubella antibody in rheumatoid patients with that in control groups of healthy persons, or patients suffering from other forms of arthritis (Chandler *et al.*, 1971; Laitinen *et al.*, 1972; Simsarian *et al.*, 1970; Deinard *et al.*, 1974). These studies have generally failed to show a difference except for those described by Patterson *et al.*, (1973 and personal communication) and Deinard *et al.* (1974). Other workers have examined antibody from RA synovial fluid cryoprecipitates, or eluates from RA synovial membranes without, however, detecting striking amounts of rubella antibody (Cremer *et al.*, 1974; Zvaifler, 1973).

However, the resolution of rubella virus infection may be protracted in some rheumatoid patients. Thus Ogra, *et al.* (1975) observed a prolonged infection with rubella virus in some JRA patients, as judged by high and persistent levels of IgM and IgG antibody to rubella virus and the demonstration of rubella antigen by immunofluorescence in smears of synovial fluid cells. These effects may be related to the higher prevalence of histocompatibility antigen HLA-B27 in JRA patients (Racheleksfy *et al.*, 1974), with its postulated immunological implications.

An association of persistent rubella infection and adult RA appears to be much less common. A serologically documented case of rubella arthritis progressing to, or associated with, classical adult RA was described by Martenis *et al.* (1968), who mentioned other patients. More recently, in Edinburgh, a 26-year-old woman has been observed who had an illness resembling rubella and subsequently developed classical RA with positive latex and SSCT tests and a raised erythrocyte sedimentation rate. A raised titre (1/2048–1/4096) of rubella antibody by haemagglutination-inhibition in both the IgG and IgM fractions of her serum was detected early in the illness. The rheumatoid state persisted for 18 months after onset of symptoms when there was a resolution of the symptoms and signs accompanied by a fall in the level of IgM rubella antibody (Duthie *et al.*, unpublished).

Other investigators have approached the problem by trying to show rubella virions or other gene products, or interference by rubella virus, in RA synovial cells, usually fibroblasts. Thus Grayzel and Beck (1970), Smith and Hamerman (1969), and Ford and Oh (1965) found that RA synovial fibroblasts were more resistant to infection with rubella or Newcastle disease viruses than non-RA fibroblasts. This was construed as an intrinsic viral interference, but

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Patterson *et al.* (1975) and Clarris *et al.* (1974) have shown that with rubella virus and Newcastle disease virus the apparent resistance of RA synovial fibroblasts is due to a thicker capsule of hyaluronic acid which hinders viral absorption; it can be removed by pretreatment with hyaluronidase.

The mechanism of chronic cellular infection by rubella virus is perhaps not completely understood but presumably involves persistence of infectious virions or viral RNA as cytoplasmic entities transmitted at cell division. Attempts to show infectious rubella virus in RA synovial fibroblasts have been negative by conventional cell culture inoculation, or co-cultivation, as have attempts to show incorporation of radiolabelled uridine into components of fibroblasts with the sedimentation characteristics of rubella RNA or virions (Grayzel, 1973; Person *et al.*, 1973; Norval and Marmion, 1976). Although there appear to be no analogies from cell systems persistently infected with rubella virus, the formal, if slightly heterodox possibility remains that rubella infection of RA synovial cells might take the form of the expression of viral antigens without infectious virions, given, for example, persistence of genetic information in the form of an integrated DNA copy of the RNA viral genome (Sato *et al.*, 1976).

In this context a recent, brief preliminary report by Patterson *et al.* (1973) was therefore of much interest. These workers, using ^{51}Cr release from cells, complement, and a rubella antiserum from a hyperimmunized rhesus monkey showed (presumptive) rubella antigens on 4 of 6 strains of RA synovial fibroblasts and on a chronically infected cell strain from an infant with the rubella syndrome. Control fibroblasts were negative. This important observation clearly required confirmation, if possible, by other serological techniques. In this paper we report the negative results of attempts to show rubella virus-coded antigens on RA and non-RA synovial fibroblasts by immunofluorescence or radioimmunoassay, and also summarize our own surveys of sera and synovial membrane eluates from RA and non-RA patients for rubella antibody.

Materials and methods

SYNOVIAL FLUID AND MEMBRANE CULTURES FROM RA OR NON-RA PATIENTS

Fibroblasts from synovial fluids were established by mixing equal volumes of fluid and Eagles Complete Medium (1959 modification). No serum was used at this stage. After 5 days the fluid was replaced by Eagles Complete Medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% fetal calf serum (Tissue Culture Services). Some of the primary cultures were also incubated in the presence of sodium aurothiomalate 10 μg/ml (Myocrisin, 45% metallic gold, May and Baker, Dagenham, England) and hydrocortisone 36 μg/ml (Sigma). Sodium aurothiomalate is concentrated within phagocytic cells and inhibits their lysosomal enzyme activity (Persellin and Ziff, 1966); it has also been shown to inhibit the cytotoxic and antitumour cell activity of macrophages (McBride *et al.*, 1975; Ghaffar *et al.*, 1976). Synovial fibroblasts with 'nonself' or neoantigens destined for destruction by macrophages might thereby be preserved for examination. Glucocorticosteroids have been reported to increase proliferation of adult human fibroblasts *in vitro* and reduce the rate of hyaluronic acid formation (Castor, 1965).

Fibroblasts from synovial membrane were obtained by overnight trypsinization of synovial tissue (0·25% trypsin in Dulbecco solution) at 37°C and transfer of the free cells into Eagles Complete Medium containing penicillin and streptomycin with 10% fetal calf serum. The monolayers of fibroblasts from either source were then propagated by trypsinization. Some of the features of these fibroblast cultures, marked A to N, in particular their examination for the presence of leucoviruses, are described elsewhere (Norval and Marmion, 1976). Additional fibroblasts have been used in this study and are labelled II-XXI.

VIRUSES

The HPV-77 rubella virus, vaccine strain and a recent isolate strain 'Thomas', were kindly supplied by Drs. Gould and Freestone of the Wellcome Research Laboratories (Beckenham).

INFECTED AND NONINFECTED CELLS FOR CONTROLS IN SEROLOGICAL TESTS

A persistent infection was established in the LCC-MK_2 cell line (American Cell Type Collection) with the HPV-77 rubella virus strain and kept on 199 medium (Wellcome) with 2% heat inactivated (56°C) fetal calf serum (FCS). The persistence and growth curve of rubella virus in these cells has been described (Maassab *et al.*, 1964) and their use for indirect immunofluorescence has been evaluated (Brown *et al.*, 1964). The infected cells change morphology, becoming sparser, fatter, and more elongated compared with the uninfected parent cell. After an initial 3 weeks they were subcultured every 2 weeks. The presence of rubella virus was confirmed by indirect immunofluorescence (Lennette *et al.*, 1967) and by labelling with ^{3}H-uridine (Norval and Marmion, 1976). For lytic infection the Thomas or HPV-77 strain of rubella virus was absorbed on to BHK-21 cells in suspension as described by Lennette

and Schmidt (1968) and maintained in Eagles Complete Medium with 2% FCS. For the detection of rubella membrane antigens the virus strains 'Thomas' and HPV-77 were propagated in BHK-21 with Complete Eagles Medium, supplemented with 1% FCS. The cells were used 4 days after being infected.

LYMPHOCYTES

Peripheral blood and synovial fluid lymphocytes were separated on Triosil-Ficoll gradients. Synovial fluids were treated with 80 units/ml hyaluronidase for 30 min at 37°C before separation on the gradient. Lymphocytes were used at a concentration of 10^7 cells/ml for the immunofluorescence test.

MYCOPLASMA TESTS

All cultures of synovial fibroblasts or continuous lines were mycoplasma-free except for A from which *Mycoplasma orale* was cultured.

ANTISERA

Antisera to HPV-77 rubella were produced by multiple injections of 10× concentrated supernatant (polyethylene glycol) from RK_{13} culture (199 medium supplemented with 5% rabbit serum free of rubella antibody) into New Zealand White rabbits. Initially 5 ml of fluid was given subcutaneously and 1 ml intravenously, followed by 4-weekly injections of 1 ml intravenously (Plotkin, 1969). 2 weeks after the last inoculation the rabbits were bled and their sera tested by the haemagglutination-inhibition test (HAI) for rubella antibodies; this schedule was repeated until their sera had HAI titres of 1/1024-2048. These high titre sera were then absorbed with human liver powder (acetone-treated) and Bentonite particles coated with fetal calf serum (Goodman and Bozicevich, 1964).

CONJUGATES

For indirect immunofluorescence sheep antirabbit immunoglobulin conjugated with fluorescein isothiocyanate (Wellcome Reagents) was used, diluted 1/10 in 20% beef brain saline to reduce nonspecific staining. Goat antirabbit immunoglobulin from Nordic Diagnostics Ltd. was used in the radioimmunoassay. It was fractionated on DEAE-cellulose DE52 (Reif, 1969); the resulting goat IgG was labelled with ^{125}I by the chloramine-T method (Hunter, 1974). The ^{125}I was added at a concentration of 500 μCi/50 μg protein.

IMMUNOFLUORESCENCE

The indirect immunofluorescence test was performed on acetone-fixed (10 min at room temperature) synovial cells grown on coverslips (6×22 mm) (Lennette *et al.*, 1967; Schmidt *et al.*, 1966). Some were treated to remove the hyaluronic acid produced by fibroblasts, by including 80 units/ml bovine hyaluronidase (Sigma, Type I) in the complete medium as described by Clarris *et al.* (1974). Each test included persistently infected $LLC\text{-}MK_2$ cells as a positive control.

To detect rubella virus membrane antigens the virus infected cells were used unfixed, either in suspension (lymphocytes) (Yoshiki *et al.*, 1974), or as fibroblasts grown on coverslips (M. Haire, personal communication). Later, to minimize cell detachment, fixation in 4% formol-saline for 10 min was adopted (K. B. Fraser and M. Haire, personal communication), followed by a 30-min wash in phosphate-buffered saline (PBS). After washing, and without drying, the antigen test was carried out using the same method as described for the acetone fixed cells.

SOLID PHASE RADIOIMMUNOASSAY

In the solid phase radioimmunoassay for antigen (described by Forghani *et al.*, 1974) the virus infected cells and synovial fibroblasts were grown on the bottom of glass vials (controlled neck shell vials, 9·5/0·5×48/50, Johnsen and Jorgensen, London) in 0·4 ml of media. The concentration of the cell seed was 20 000/ml media. After 4 days' incubation at 37°C the cultures contained approximately 50 000 cells per vial. After removal of the growth medium the cells were rinsed with 1 ml of PBS, and then fixed in cold acetone for 10 min while still wet. The antiserum dilutions were made in PBS supplemented with 2% FCS. 0·1 ml was then added to each vial and incubated for 2 hours at 37°C. The contents of the vials were then aspirated and they were washed 4 times in PBS. The ^{125}I conjugate was diluted to contain 60 000 counts/100 s in 0·1 ml. This quantity was added to each vial and they were incubated for 80 min at room temperature. The contents were aspirated and the vials were again washed 4 times. The residual radioactivity was assayed on a gamma counter (Wallac-LKB counting system).

ELUTION OF IMMUNOGLOBULINS FROM SYNOVIAL MEMBRANES

The method followed that of McCormick *et al.* (1971). Synovial membranes obtained at operation were finely chopped and were repeatedly washed with PBS pH 7·2. The fluids were checked for adsorption at 280 nm; when this indicated minimal protein, glycine buffer pH 2·5 10 ml/g wet tissue was added and stirred at 4°C for 2 hours. The 'eluates' were centrifuged immediately at 100 000 *g* for 1 hour to remove any dissociated 'antigen'. pH was then adjusted to 7·2 with solid TRIS buffer and the

fluid dialysed for 72 hours against PBS pH 7·2. Sodium azide at 0·1% concentration was added and the 'eluates' stored at 4°C until required for rubella HAI tests. The fluid from the last wash was kept and tested in parallel.

Results

ATTEMPTS TO DETECT RUBELLA VIRUS ANTIGEN BY IMMUNOFLUORESCENCE

(a) Acetone fixed cells grown on coverslips

The LLC-MK_2- cells persistently infected with HPV-77 virus showed a granular cytoplasmic staining that sometimes extended round the nucleus, but was mainly located on one side, in the form of bright cytoplasmic inclusions. This type of staining was detected in about 80% of the persistently infected cells; the number differed according to the number of days after trypsinization and the antiserum dilution used. Such granular cytoplasmic staining was not observed in synovial fibroblasts from any source, either with or without hyaluronidase pretreatment (Table 1). The synovial fibroblasts were prepared from 8 classical or definite cases of RA, 7 of osteoarthrosis, and one other form of inflammatory joint disease (non-RA).

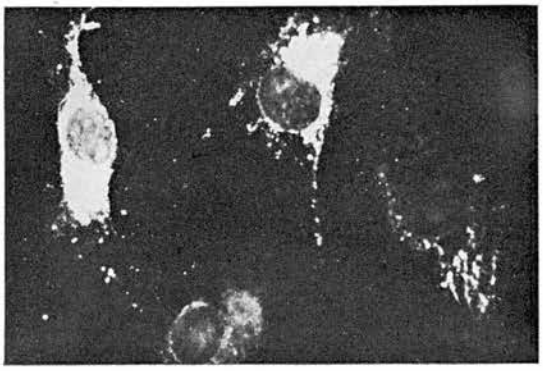
(b) Membrane antigens

It was possible to detect membrane antigens on unfixed BHK-21 cells, infected with rubella virus and grown on glass coverslips and also on virus infected cells fixed in formol-saline (Fig. 1). The latter technique was used on the collection of synovial fibroblasts from different sources and all gave negative results irrespective of pretreatment with hyaluronidase (Table 1). It will be noted from Table 1 that whereas cytoplasmic and membrane staining was readily shown with acute infection of BHK fibroblasts, the LLC-MK_2 cells chronically infected with HPV-77 showed only a weak or no membrane staining.

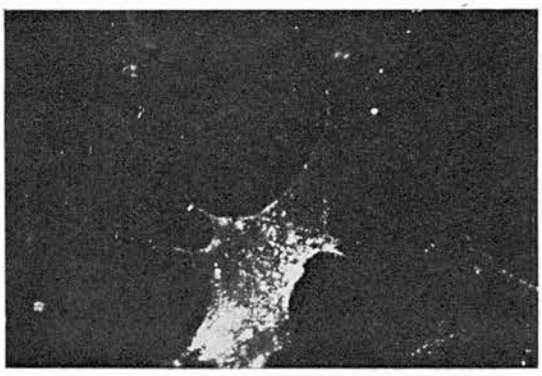
Table 1 *Examination of synovial fibroblasts by immunofluorescence for the cytoplasmic or membrane antigens of rubella virus*

Culture		*Diagnosis*	*Source*	*Tissue*	*Treatment of fibroblasts*	*Immunofluorescence cytoplasmic/membrane*	
A	P10	RA for 3 yr	R70 (Dr. Hamerman)	SM	—	—	—
	P10				H	—	—
D	P24	OA	N104 "	SM	—	—	..
	P24				H	—	..
E	P12	RA for 14 yr	17/74 (W. Neill)	SM	H	—	±
F	P14	RA for 14 yr	20/74 "	SM	—	—	—
	P14				H	—	±
G	P11	OA for 4 yr	23/74 "	SM	—	—	±
	P11				H	—	±
H	P20	RA for 4 yr	24/74 "	SM	—	—	—
	P20				H	—	±
I	P15	Polyarthritis (non-RA)	25/74 "	SM	—	—	—
	P15				H	—	—
J	P19	RA for 12 yr	27/74 "	SM	—	—	—
	P19				H	—	—
K	P16	OA for 8 yr	33/74 "	SM	—	..	—
	P16				H	—	—
M	P10	RA	42/74 "	SM	—	—	..
	P10				H	—	..
N	P13	OA for 3 yr	—(Virus research group)	SM	—	—	—
	P13				H	—	—
II	P4	OA	— "	SF*	—	—	..
	P4	4 yr			H	—	..
III	P6	RA	— "	SF**	—	—	..
	P6	22 yr			H	—	..
IV	P6	RA	— "	SF**	—	—	..
	P6	4½ yr			H	—	..
VI	P4	OA	— "	SF**	—	—	..
	P4	50 yr			H	—	..
VII	P3	OA	— "	SF*	—	—	..
	P3	8 yr			H	—	..
	P3			SF**	—	—	..
	P3				H	—	..
HPV-77-LLC-MK2		P14	..	Monkey	..	++	—
LLC-MK2		P12	..	kidney	..	—	—
Thomas-BHK-21		P8	..	Hamster	..	+++	++
BHK-21		P8	..	kidney	..	—	—

SM=synovial membrane; SF*=synovial fluid culture was isolated with hydrocortisone (36 μg/ml); SF**=synovial fibroblasts were isolated in the presence of hydrocortisone and sodium aurothiomalate (10 μg/ml); H=hyaluronidase (80 units/ml). ± to +++=increasing degrees of immunofluorescence. —= negative immunofluorescence. ··=not tested or no treatment. P=passage level



(*a*)



(*b*)

Fig. 1 (*a*) *Cytoplasmic accumulations of rubella virus antigen in BHK-21 cells, fixed with acetone and stained with rubella antiserum (R3/HPV-77/6-14.3.75) at 1/5 dilution.* (*b*) *Membrane immunofluorescence of rubella virus in BHK-21, fixed with 4% formol-saline and stained with the same serum.*

Separated lymphocytes from synovial fluid and peripheral blood were treated with hyperimmune rabbit rubella antiserum (R3/HPV-77/6–14·3·75) at 1 : 10 dilution as for membrane antigens. There was no membrane immunofluorescence with 8 lots of synovial lymphocytes from RA patients and one JRA patient. In 2 instances the peripheral blood lymphocytes from the same patient were also treated, but with negative results.

ATTEMPTS TO DETECT RUBELLA VIRUS ANTIGEN BY RADIOIMMUNOASSAY

Solid-phase radioimmunoassay will detect viral antigens in infected cells (Hayashi *et al.*, 1972, 1973; Forghani *et al.*, 1974) and its sensitivity has been shown to be greater than that of immunofluorescence (Hayashi *et al.*, 1973–74). So far as we are aware it has not been applied to the detection of rubella antigens; it was felt that a test of greater sensitivity than immunofluorescence should be used in further attempts to validate the results of Patterson *et al.* (1973), particularly in view of the failure to show membrane antigens on HPV-77-LLC-MK_2 by immunofluorescence (Table 1).

Calibration of radioimmunoassay

The rubella (HPV-77) antiserum (R3/HPV-77/6–14.3.75) from the hyperimmunized rabbit R3 and its preimmunization serum (R3/N2), were titrated by radioimmunoassay on BHK 21 cells acutely infected with the strain 'Thomas' and LLC-MK_2 cells chronically infected with HPV-77. The preimmunization and the antiserum were absorbed with human liver powder, LLC-MK_2 cells, and human diploid fibroblasts; half-log dilutions from 1/30 to 1/100 000 of the sera were layered on monolayers on the bottom of glass vials. Goat IgG labelled with ^{125}I and containing the antibody against rabbit IgG was used at a dilution giving 60 000 counts/100 s in 100 µl. The resulting titration curves of the two sera on infected and uninfected BHK 21 cells is shown in Fig. 2 and on infected and uninfected LLC-MK_2 in Fig 3. In each instance the number of counts bound to infected cells treated with antiserum R3/HPV-77/6-14.3.75 was substantially in excess, over the range of dilutions from 1/30–1/3000, when compared with the same serum on uninfected cells, or the preimmunization serum on

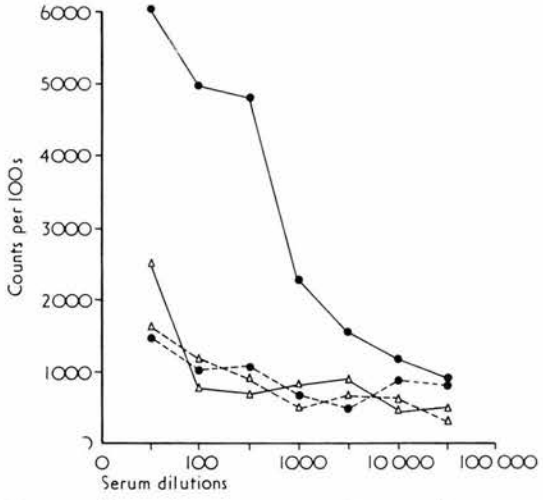


Fig. 2 *Effects of a concentration of intermediate serum on binding with rubella antigens.* ●, *Thomas-rubella infected BHK-21 cells;* △, *control BHK-21 cells;* —, *R3/HPV-77/6–14.3.75 hyperimmune rubella antiserum;* ----, *R3/N2 preimmunization serum.*

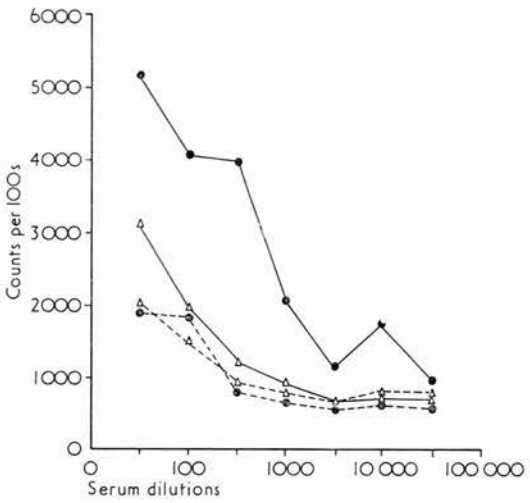


Fig. 3 *Radioimmunoassay with hyperimmune anti-rubella rabbit serum.* ^{125}*I-labelled goat antirabbit IgG was used at 60 000 counts/100 s.* ●, *chronically infected LLC-MK2 cells with HPV-77 rubella;* △, *control LLC-MK2 cells;* —, *hyperimmune antirubella rabbit serum R3/HPV-77/6-14.3.75;* ----, *preimmunization serum R3/HPV-77/N2.*

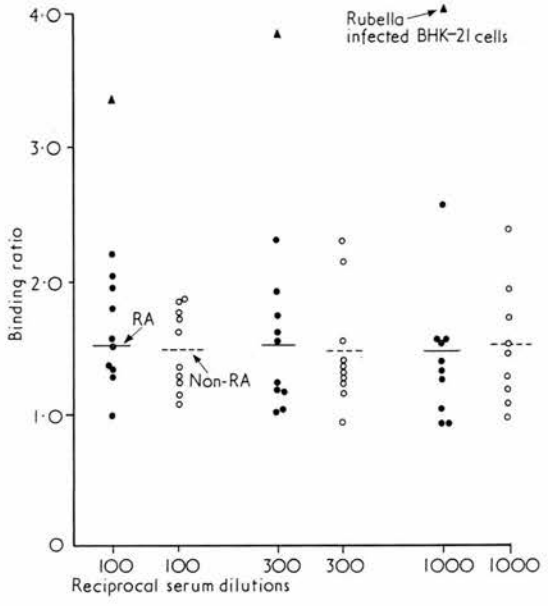


Fig. 4 *The binding ratios for each serum dilution (c/100 s for hyperimmune serum R3/HPV-77/6-14.3.75 divided by c/100 s for the preimmunization serum) are plotted in 2 groups: RA fibroblasts* (●), *and non-RA fibroblasts* (o) *include osteoarthrosis and other forms of inflammatory joint diseases.*

infected or uninfected cells. Inspection of the curves suggests a titration end point in the region 1/1000–1/3000 which approximates to the titre of 1/2048 of the serum measured by HAI before absorption.

An optimal dilution of 1/300 of the antiserum was evident on the basis that the distance separating the antiserum and control curves was greatest at that dilution. Activity at that antiserum dilution on the BHK 21 cell system could be expressed either (i) as a binding ratio of counts/100 s of serum dilution on virus infected cells divided by counts/100 s on uninfected cells—giving values, respectively, of 6·3 for the immune serum and 1·3 for the preimmune serum—or (ii) as a binding ratio, counts per 100 s antiserum/counts per 100 s preimmune serum on the same cells, with values respectively of 4·5 and $<1·0$. The chronically infected HPV-77-LLC-MK$_2$ cells and the controls gave binding ratios of 4·9 and 1·2 by method (ii). These cells had been infected for one year at that time and were in the 22nd passage (3·5% of cells showed immunofluorescence at that stage compared with about 80% after the initial infection).

Synovial fibroblasts were grown on the bottom of glass vials and exposed to 1/100, 1/300, and 1/1000 dilutions of the antiserum, or of the corresponding preimmunization serum, and the binding ratios were calculated by method (ii). Fig. 4 shows the distribution of binding ratios for 10 RA synovial fibroblasts and 10 non-RA fibroblasts (A–N and II–VII, Table 1), at the 3 dilutions of serum R1/HPV-77/4-7.1.75, and also the ratios obtained with rubella virus infected BHK cells. There was little difference in the distribution of counts bound between RA synovial fibroblasts on the one hand and non-RA fibroblasts on the other. However, it was known that despite attempts to use a homologous system the antiserum had some activity against calf serum due to problems of residual contamination with calf serum of the antigen for immunizing the rabbit. Because of this and also because the collection of synovial fibroblasts was at a high passage level, some fresh isolates (VIII–XXI) of fibroblasts were established and tested with R29/N1 (preimmunization serum) and R29/Thomas/2-23.9.75 (hyperimmune) rabbit antiserum (HAI titre 1:2048) (see Table 2).

Five samples were used at each dilution and standard deviations were also calculated. Of 11 cultures of fibroblasts from patients with definite RA only one culture had slightly raised binding ratio (XIX P4). However, a raised binding ratio was also found in one culture out of six non-RA fibroblasts (N P17). These results do not support the view that rubella antigens are expressed in synovial fibroblasts from rheumatoid patients.

Table 2 *Examination of hyaluronidase-treated synovial fibroblasts from patients with rheumatoid arthritis, and other joint conditions, for rubella antigens detectable by radioimmunoassay with pre- and postimmunization sera from rabbit 29 given the 'Thomas' strain of rubella virus*

Culture no.		*Diagnosis*	*Serum dilution*	*Counts bound (± SD) with*		*Binding ratio*
				Rubella antiserum (R29/THOMAS/ 2–23.9.75)	*Preimmunization serum (R29/N1)*	
G	P17	OA (4 yr)	1 : 100	529±238	415± 97	1·27
			1 : 300	449± 48	371± 73	1·35
			1 : 1000	297± 26	326± 43	<1
I	P19	Polyarthritis (non-RA)	1 : 100	597±110	403± 36	1·48
			1 : 300	437± 57	408± 58	1·07
			1 : 1000	317± 29	310± 8	1·02
N	P17	OA (3 yr)	1 : 100	628± 84	323± 24	1·94
			1 : 300	381± 62	281± 22	1·36
			1 : 1000	309± 45	235± 32	1·31
V	P8	RA (2½ yrs)	1 : 100	699± 51	918± 54	<1
			1 : 300	512± 39	732± 73	<1
			1 : 1000	387± 42	525± 53	<1
VIII	P7	OA (8 yr)	1 : 100	436± 60	577± 63	<1
			1 : 300	336± 63	457± 46	<1
			1 : 1000	253± 54	335± 31	<1
IX	P6	RA (3 yr)	1 : 100	243± 14	229± 37	1·06
			1 : 300	186± 23	261± 46	<1
			1 : 1000	180±111	227± 37	<1
X	P7	RA (6½ yr)	1 : 100	465± 64	315± 22	1·48
			1 : 300	315± 39	288± 36	1·09
			1 : 1000	309±172	273± 32	1·13
XI	P7	non-RA (7 yr)	1 : 100	302± 26	407± 44	<1
			1 : 300	211± 10	260± 19	<1
			1 : 1000	152± 29	170± 10	<1
XII	P3	RA (7 yr)	1 : 100	324± 50	518± 97	<1
			1 : 300	252± 31	359± 33	<1
			1 : 1000	218± 47	209± 46	1·04
XIII	P7	RA (6 yr)	1 : 100	450± 63	760± 67	<1
			1 : 300	316± 67	533±181	<1
			1 : 1000	275± 49	340± 49	<1
XIV	P5	Prob. RA (6 yr)	1 : 100	527±131	506± 28	1·04
			1 : 300	331± 31	367± 45	<1
			1 : 1000	277± 41	264± 15	1·05
XV	P4	RA	1 : 100	257± 43	346± 95	<1
			1 : 300	256± 71	313± 44	<1
			1 : 1000	219± 33	227± 60	<1
XVI	P7	RA	1 : 100	494± 14	505±119	<1
			1 : 300	346± 61	321± 24	1·08
			1 : 1000	252± 18	239± 31	1·05
XVII	P7	RA	1 : 100	1016±180	1365±196	<1
			1 : 300	829±264	1211±294	<1
			1 : 1000	818±108	1003±168	<1
XVIII	P5	RA	1 : 100	454± 80	503± 64	<1
			1 : 300	339± 24	437± 35	<1
			1 : 1000	249± 32	318± 58	<1
XIX	P4	RA (21 yr)	1 : 100	459± 37	295± 30	1·56
			1 : 300	374± 57	218± 20	1·72
			1 : 1000	230± 19	171± 22	1·35
XX	P3	RA (10 yr)	1 : 100	505± 35	721± 43	<1
			1 : 300	361± 78	512± 29	<1
			1 : 1000	261± 40	398±117	<1
XXI	P5	Psoriatic arthropathy (4 yr)	1 : 100	526± 91	614± 39	<1
			1 : 300	429± 71	435± 46	<1
			1 : 1000	395± 35	423±132	<1
Control						
BHK-21 infected with Thomas strain of rubella virus			1 : 100	1935±141	884± 29	2·19
			1 : 300	1895±129	803± 58	2·36
			1 : 1000	1313±132	702±110	1·87

EXAMINATION OF RA SYNOVIAL MEMBRANES FOR BOUND ANTIBODY TO RUBELLA VIRUS

If the antigen-antibody reaction initiating the rheumatoid process were a combination between rubella virus-coded or induced antigens in synovial cells, and locally produced antibody, it might be expected that material eluted from RA membranes under conditions that dissociate antigen-antibody complexes would contain substantial amounts of antibody. This method has been used for example to demonstrate antibody to nucleic acid or Gross leukaemia virus in the kidneys of NZB mice or those chronically infected with lymphocytic choriomeningitis virus (Mellors *et al.*, 1971; Oldstone and Dixon, 1971). This aspect has been investigated in collaboration with Dr. J. McCormick. Synovial membrane eluates from 14 patients (13 RA and 1 non-RA) which contained protein demonstrable by adsorption at 280 nm were tested for a variety of viral antibodies, including those against rubella. All were negative at a dilution of 1/4 in the standard rubella HAI test. This approach is being pursued further with estimations of total immunoglobulins, rheumatoid and antinuclear factors, as well as other specific viral antibodies in the eluates (J. McCormick and H. Hart, to be published).

Table 3 *Distribution of haemagglutination-inhibiting (HAI) antibody to rubella virus among patients with rheumatoid arthritis and other arthritic conditions. (Patients with antibody titres ⩾64 were regarded as positive)*

Category	*No in category with rubella HAI antibody titres of*		*Total*
	⩽32	*⩾64*	
Rheumatoid arthritis (RA)	49	31	80
non-RA	42	20	62
Total	91	51	142

EXAMINATION OF RA AND NON-RA PATIENTS FOR CIRCULATING ANTIBODY TO RUBELLA VIRUS

In view of the differences in prevalence rates and geometric mean titres of antibody to rubella virus found in RA patients and *healthy* individuals by Patterson *et al.* (1973 and personal communication), we record in Table 3 the results of testing RA and non-RA patients for rubella HAI antibody by standard methods; these figures are extracted from a larger survey done by Susan Baker and workers at the Northern General Hospital and covering a variety of viruses and immunoglobulin fractions. As in numerous previous investigations there was no significant difference between the frequency or levels of rubella antibody in the two groups ($P=0{\cdot}72$).

Discussion

There are several ways in which (hypothetically) rubella virus (or other viruses) might be involved in RA; (i) persistence in the A or B lining cells, vascular endothelium of the synovium, or in cartilage, (ii) generation of antibody cross-reacting with synovial antigens as a result of viral infection at a distant site, (iii) lodgement in the synovium of immune complexes of virus and antibody generated elsewhere in the body, and (iv) viral infection of synovial lymphocytes or macrophages with impairment of homoeostatic mechanisms limiting macrophage action (see also Marmion, 1976; Morley, 1974).

The results reported in this paper, together with those of Person *et al.* (1973) and Norval and Marmion (1976), cover a number of aspects of (i) and (ii) for rubella virus, but as the virus has been found in the cartilage chondrocytes of congenitally infected rabbits (London *et al.*, 1970; Smith *et al.*, 1973) and as this tissue has not been covered in our studies of human RA, investigations of the joint *per se* are not complete. Possibility (iii) would probably not be covered for rubella virus by the examination of synovial membrane eluates as the complexes could be inside phagocytic cells, or, if free in interstices of the membrane, lost during the washing process. As for possibility (iv), rubella infected lymphocytes have been shown to have a decreased response to phytohaemagglutinin stimulation (McMorrow *et al.*, 1974; Vesikari and Biumovici-Klein, 1975) and decreased responsiveness to phytohaemagglutinin and other antigens has been observed in RA synovial lymphocytes (G. S. Panayi, personal communication; Hedberg *et al.*, 1971; Astorga and Williams, 1969). However, our limited observations on synovial lymphocytes do not indicate that this anergy is due to the presence of rubella virus coded antigens in the lymphocyte membrane.

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Case report

Rheumatoid polyarthritis after rubella

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SUMMARY A 24-year-old woman developed persistent polyarthritis indistinguishable from rheumatoid arthritis after rubella. The arthritis persisted for approximately 30 months and was associated with high levels of antibody to rubella virus and with rheumatoid factor. The antibody titres declined *pari passu* with clinical improvement which progressed to complete resolution. Fractionation of serial serum specimens showed a substantial and persistent IgM antibody response to rubella virus. Rubella antigen was not demonstrated in the synovial exudate.

The association of a transient, self-limiting, arthritis with natural rubella infection or following immunisation with attenuated rubella virus is well documented (e.g. Moylan-Jones and Penney, 1962; Kantor and Tanner, 1962; Chambers and Bywaters, 1963; Yanez *et al.*, 1966; Thompson *et al.*, 1971). Less commonly, the arthritis relapses (A. J. Tingle *et al.*, personal communication, 1977) or causes longer disability—'catchers crouch syndrome' (Spotswood *et al.*, 1977).

A causal connection between rubella virus infection and juvenile or adult rheumatoid arthritis (RA) has been sought by various workers during the past 20 years but with generally negative results (see Hart and Marmion, 1977). Nevertheless, the resolution of rubella virus infection appears to be protracted in some patients with RA. Thus, Ogra *et al.* (1975) postulated prolonged infection with rubella virus in some patients with juvenile RA on finding high and persistent serum levels of IgM and IgG rubella antibody and rubella antigen in smears of synovial fluid cells by immunofluorescence. Rubella virus has also been isolated from synovial fluid after natural infection (Hildebrandt and Maassab, 1966) and after immunisation with rubella vaccine (Weibel *et al.*, 1969). In another report by Ogra and Herd (1971), rubella virus was isolated from the synovial fluid of 3 previously healthy children 3–4 months after immunisation. Those authors also described a child with a pre-existing chronic polyarthritis who developed repeated episodes of joint effusion for 3 months after immunisation but without virus isolation.

In adults there have been occasional reports suggesting progression of rubella arthritis to typical RA. Martenis *et al.* (1968) describe one patient in detail and mention another less adequately documented case (Riddell, 1962). Perhaps the immunological abnormalities that characterise RA may be precipitated, or accentuated, in some patients by a rubella virus infection, but the infrequent association observed between rubella infection and RA may be more of a chance inter-relating event than true cause and effect.

Because of these associations, we describe the case of a 24-year-old woman who had an illness resembling rubella and subsequently developed an RA-like condition with positive latex and sheep cell agglutination tests and a raised erythrocyte sedimentation rate (ESR). This state lasted for more than 24 months and was accompanied by high levels of antibody to rubella virus. Then her polyarthritis resolved in step with a fall in antibody levels against rubella virus, particularly in the IgM fraction.

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Case history

A 24-year-old woman, previously healthy, developed a sore throat and coryza in mid-June 1970. Two days later an itchy, erythematous rash appeared on her

arms, legs, and trunk. 3 days later she developed a flitting monarthritis. The affected joints were painful, obviously swollen and inflamed, and the attacks lasted several hours. There was no morning stiffness. Tetracycline, penicillin, and cotrimoxazole were given in turn but were discontinued because of side effects.

The flitting joint symptoms and the rash persisted; the latter was variable in severity, becoming more evident towards evening. On July 9th she was referred to hospital where a provisional diagnosis of rheumatic fever was made. A throat swab yielded scanty β-haemolytic streptococci but the antistreptolysin O titre was less than 125 Todd units; the RA latex test was negative but her ESR was 40 mm/h. Radiographs of the chest, hands, and wrists showed no abnormality.

On admission to hospital on July 23 she was pyrexial (temperature up to 39·2°C) with a variable rash on her back, arms, and legs. Posterior cervical lymph nodes were enlarged, there was a small corneal ulcer with marked conjunctivitis in the left eye; there was also a monilial infection of the vulva. Several joints were painful on movement, although not swollen.

Electrocardiogram, chest *x*-ray, urinalysis, and routine biochemical tests showed no abnormality but the ESR had increased to 123 mm/h. The RA latex fixation and antinuclear factor (ANF) tests were negative and LE cells were not demonstrated. Serological tests for brucellosis, infectious mononucleosis, and syphilis were negative. Rubella was suspected although there was no known contact; a serum sample taken on July 24—6 weeks after the onset of sore throat and rash—was eventually reported to have a haemagglutination inhibition (HAI) titre of 1/512 and a CF titre of 1/32. Her pyrexia and joint symptoms settled with soluble aspirin and the corneal ulcer and monilial infection responded to local treatment. On discharge from hospital on 18 August 1970, there was no arthritis or fever but there was still some rash on her limbs.

On review as an outpatient in November 1970 she stated that she felt very well. Her hands showed the clinical appearance of RA with swelling of the proximal interphalangeal joints but radiographs showed no abnormality. Haemoglobulin was 11·4 g/dl and the ESR was normal at 12 mm/h. The RA latex and ANF tests were again negative.

On February 2, 1971 she was readmitted to the same hospital with a recurrence of polyarthritis after an upper respiratory tract infection in December 1970. Her haemoglobin had fallen to 7·0 g/dl and the ESR was considerably raised at 110 mm/h. Her condition did not improve and she was transferred on March 11 to the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh, for further investigation and treatment.

On examination there was spindling and cyanosis of the proximal interphalangeal joints and some swelling of the metacarpophalangeal joints in both hands (Fig. 1). The wrists were slightly swollen, there was some limitation of extension in the elbows and synovial swelling and effusions in both knees

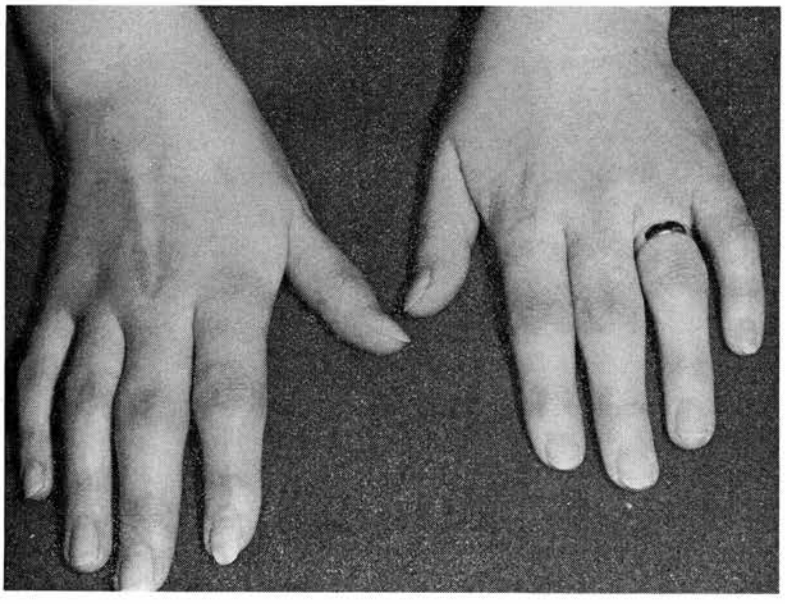

Fig. 1 *Polyarthritic changes in the patient's hands, March 1971.*

The ankles were also slightly swollen. Radiographs of the hands and wrists showed some periarticular osteoporosis but no erosions. The knees and feet were radiographically normal. A few cervical lymph nodes were palpable but there was no rash.

Haemoglobin was 8·5 g/dl and ESR was 132 mm/h. The anaemia was haemolytic in type and her serum contained a weak haemagglutinin detectable with enzyme-treated cells, and at all temperatures. The Rose-Waaler test (SSCT) was positive at 1 in 128 on March 11 but the tests for ANF and LE cells were again negative. Rubella antibody tests on March 12 and April 16 gave HAI titres of 1/1024–2048 and CF titres of 1/16.

The joint symptoms settled down with bed rest, splinting, ACTH 20 units daily, and full doses of soluble aspirin. The left knee was aspirated on two occasions and injected with hydrocortisone. The synovial fluid contained 12 500–16 000 cells/ml which were predominantly mononuclear. The anaemia was corrected by 2 g intravenous iron and by transfusion of 4 units of packed cells. On discharge on May 15 she was feeling quite well and had only minimal discomfort in her joints.

On review at outpatient department in July 1971, her haemoglobin had fallen to 7·1 g/dl and the ESR was 104 mm/h. Her joint pain and stiffness had returned with large effusions in both knees. She was therefore readmitted on July 18 and received a further transfusion of packed cells and started treatment with rifampicin 600 mg daily. She was discharged on the 23rd without further treatment but was seen regularly as an outpatient thereafter.

During the following 16 months she continued to complain of variable joint pain and stiffness, moderate to severe anaemia persisted despite oral iron, and the ESR remained raised. After 6 months' continuous therapy without obvious benefit, rifampicin was withdrawn in February 1972. Recurrent effusions in her knees were aspirated on six occasions between September 1971 and June 1972. Mononuclear cells were less predominant in these samples of synovial fluid than in the specimens obtained initially in May 1971, and 30–55% polymorphonuclear leucocytes were seen. In cytosmears of synovial fluid cells intracellular inclusions of IgG and IgM were demonstrated by immunofluorescence.

By November 1972 the haemoglobin had increased to 14·4 g/dl, the ESR had fallen to 4 mm/h, and the serological tests for rheumatoid factor were negative. She felt very well and her joints appeared normal apart from a small effusion in the left knee. By March 1973 she was symptom-free and all signs of arthritis had disappeared. When seen in December 1973 she was 4 months pregnant and had no joint symptoms. A healthy baby (her second child) was delivered in May 1974, without any post-partum recurrence of her joint symptoms. She was last seen on January 19, 1977 when she stated that she had been well until 3 months previously when she had had a transient recurrence of pain and swelling in her right knee which her general practitioner had attributed to a 'housemaid's knee'. This settled down with a supporting bandage and has not troubled her since then. There were no residual signs of arthritis in any of her joints (Fig. 2) and radiographs showed

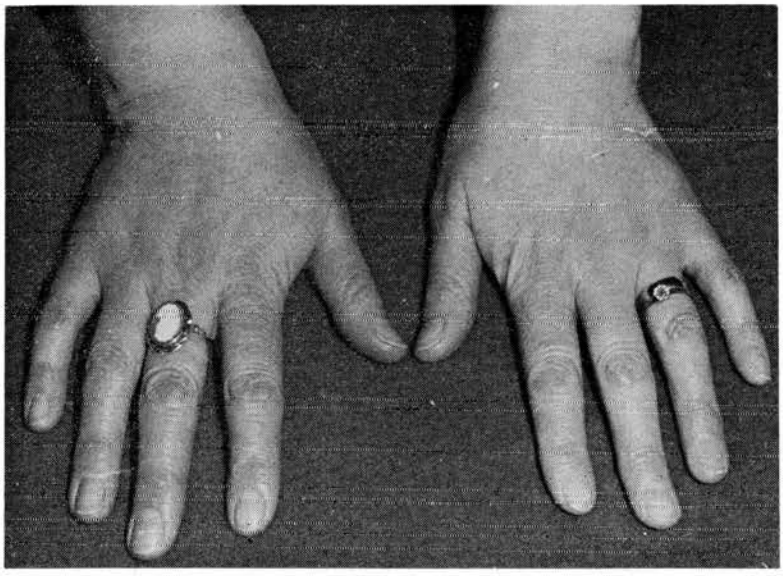

Fig. 2 *The patient's hands, January 1977.*

no abnormality in her hands, wrists, knees, and feet. Tests for rheumatoid factor and antinuclear factor were negative, haemoglobin and ESR were normal, and the rubella HAI and CF titres were 128 and 32 respectively. The course of the illness and serological findings are summarised in Fig. 3.

VIROLOGICAL INVESTIGATIONS

Tests for rubella antigens in synovial fluid cells

Specimens of synovial fluid from the knees obtained on September 8, 1971, January 4 and June 28, 1972 were examined for rubella antigen by binding of fluorescein-labelled and radiolabelled antirubella antibody. A concentrated IgG fraction (rubella HAI titre 1/512) was prepared by DEAE-cellulose chromatography from a sample of the patient's serum obtained on October 6, 1971. Aliquots were labelled with fluorescein isothiocyanate by the dialysis method (Clark and Shepard, 1963) and with ^{125}I by the iodine monochloride method (McFarlane, 1958).

The first two samples of synovial fluid were maintained in Eagle's medium/10% fetal calf serum in Leighton tubes for 33 and 20 days respectively. The 'flying' coverslips were then removed, fixed in cold acetone, and incubated with fluorescent or radiolabelled IgG antirubella antibody but no specific binding was observed. (These two samples of synovial fluid, and others taken early in the illness, were subsequently lost by refrigerator failure and were not available for attempted isolation of virus.)

The third specimen of synovial fluid (28/6/72) was examined before culture. Suspensions of washed synovial cells were incubated with both reagents at 4°C. Acetone-fixed cytosmears of fresh cells were also incubated with the FITC-labelled antirubella IgG but no specific binding was demonstrated in either system.

Attempted isolation of rubella from synovial fluid

Synovial fluid (23/8/72) for culture was taken at the time of persisting rubella IgM in patient's serum and

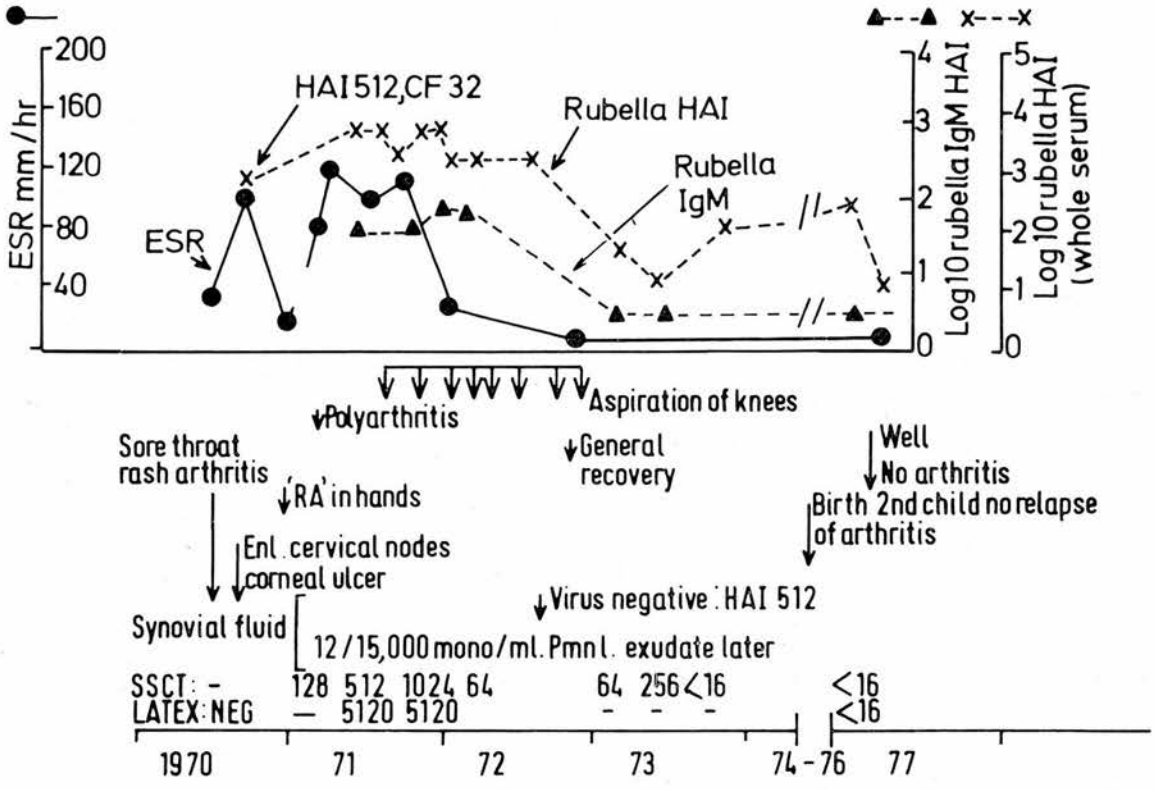

Fig. 3 *Rubella antibody and viral studies in relation to other laboratory investigations and the general clinical picture in the patient aged 24.*

contained substantial amounts of rubella antibody (see serological section). Two virus isolation methods were used.

(*a*) *In rabbits*: The method followed that of Kono *et al.* (1969) for inoculation of pregnant rabbits with strains of rubella. Two synovial fluid specimens, both collected on August 23, 1972, previously stored in liquid nitrogen, (i) in Eagle's medium containing 10% DMSO, and (ii) as concentrated synovial fluid cells, were inoculated into ear veins of two pregnant rabbits on day 8 after the copulation. The rabbit littered 22 days later and the 10 neonates showed no signs of congenital rubella including eye changes. All neonates died within 2 days of birth; rubella virus was not isolated from their heart, liver, or brain. The rubella HAI titres in the mothers remained negative at <16.

(*b*) *In tissue culture*: The synovial cells stored in DMSO were fused with RK_{13} and Vero cells using UV-inactivated Sendai virus (Watkins, 1971) in efforts to circumvent the possible neutralising effect of rubella antibody in the fluid. Fused and control cells were split once a fortnight and observed for 2 months. At fortnightly intervals fused Vero cells were tested by immunofluorescence with rubella antiserum and antirabbit Ig-FITC as described previously (Hart and Marmion, 1977). No rubella antigens were seen in Vero cells and no cytopathic effect was detected in RK_{13}.

Rubella antibody in the patient's serum and synovial fluid

Sera. 19 samples of serum collected over a period of 68 months were available. 10 of these samples, obtained between May 1971 and February 1972, were fractionated under acidic conditions by gel filtration on Sephadex G200 columns.

Fractionation of sera by gel filtration. In initial runs, 3 ml serum were applied to Sephadex G200 columns (40 × 3 cm) equilibrated in Tris/HCl buffer (0·05 mol/l, pH 8·0). 4·0 ml fractions were collected and the optical density was recorded at 280 nm. The excluded peak 1 fractions were pooled and concentrated to approximately the original serum volume by ultrafiltration in Centriflo membrane filters (Amicon). Preliminary examination of the concentrated peak 1 fractions apparently showed IgM antibody in the rubella HAI system. However, when the fractions were subjected to immunodiffusion analysis with monospecific anti-IgM and anti-IgG antisera, significant contamination with IgG was found; presumably because of complex formation between IgM rheumatoid factor and IgG.

To overcome this problem all subsequent fractionations were carried out at pH 4·0 in glycine/HCl buffer. The individual fractions from the ascending limb of the excluded first peak were pooled, the pH was adjusted to 7·0 with 0·1 M NaOH, and the pool was concentrated by ultrafiltration. Bovine serum albumin was added to a final concentration of 1·0% to reduce denaturation of IgM. These fractions were shown to be free of IgG by immunodiffusion analysis. To check that separation of IgM from IgG was complete, IgG prepared from the patient's serum by fractionation on DEAE-cellulose (DE 52) was labelled with ^{125}I by the iodine monochloride technique, and added to the serum before fractionation. On the 4 occasions when this additional safeguard was employed (sera 3, 4, 6, & 11), the fractions in the ascending limb of the first peak were shown to be free of IgG by absence of radioactivity as well as by immunodiffusion.

The results are given in the Table; rubella HAI activity was found in the majority of the IgM fractions prepared in this way. In addition, rheumatoid factor was detected by the tube latex test at a titre of 1/5120 or greater in the IgM fraction of 4 sera tested (1, 4, 7, & 10).

The results of less satisfactory separations of IgM and IgG rubella antibody on sucrose density gradients, utilising sera taken later in 1972 and in 1973, indicate a slow fall in IgM levels (Table).

Synovial fluid. Only one specimen of synovial fluid, obtained on August 23, 1972, was tested for rubella HAI antibody and had an antibody titre of 1/512. By an indirect immunofluorescence system using BHK_{21} cells infected with rubella virus (Hart and Marmion, 1977) and an anti-IgM conjugate rubella, IgM antibody appeared to be present in this synovial fluid. However, it is known that rheumatoid factor will react with specifically bound IgG antibody in immunofluorescence systems (McCormick, 1962) and it has also been suggested that the presence of rheumatoid factor will interfere with the detection of IgM antibody to virus antigens (Shirodaria *et al.*, 1973).

OTHER INVESTIGATIONS

Lymphocyte function tests

Lymphocytes separated on a Triosil/Ficoll gradient from blood obtained on January 19, 1977 showed a normal response to phytohaemagglutinin and pokeweed mitogen. There was virtually no response to PPD or Candida antigen. No information is available on lymphocyte function during the course of her illness.

HLA phenotype: A9; B13-W40.

Discussion

The clinical picture at the onset of the illness—sore throat, enlarged cervical lymph nodes, erythematous

Table *Rubella HAI titres on whole serum and IgM fractions prepared on Sephadex G200 at pH 4·0 and on sucrose gradients*

Serum no.	*Date*	*Whole serum*				*IgM fractions separated on Sephadex G200 at pH 4·0*		*IgM fractions prepared on sucrose gradients: rubella HAI titre*
		Rubella		*SSCT*	*Latex*	*Rubella HAI titre*	*Tube latex titre*	
		HAI	*CF*					
1	13/ 5/71	512	32	512	>5120	2	5120	4
2	26/ 6/71	2048	..	512	>5120	—	—	—
3	14/ 7/71	2048	..	256	>5120	128*	—	—
4	22/ 7/71	2048	32	..	..	64*	5120	—
5	8/ 9/71	1024	..	128	..	2	—	—
6	6/10/71	2048	64	..	..	32*	—	8
7	3/11/71	2048	..	1024	>5120	4	5120	—
8	17/12/71	1024	64	..	..	64	—	—
9	4/ 1/72	2048	..	64	5120	64	—	—
10	24/ 1/72	..	..	—	..	64	5120	—
11	23/ 2/72	..	..	32	..	64*	—	—
12	26/ 4/72	1024	..	16	<20	—	—	8
13	23/ 8/72	1024	..	..	..	—	—	4
14	7/ 3/73	64	..	64	..	—	—	<2
15	6/ 6/73	16	..	256	..	—	—	<2
16	15/12/73	256	32	32	..	—	—	—
17	3/ 7/74	..	..	<16	<20	—	—	<2
18	15/ 1/75	..	..	<16	<20	—	—	—
19	19/ 1/77	128	32	<16	<20	—	—	—

— = not done; * ^{125}I–IgG added before fractionation but not detected in IgM fraction.

rash, monarthritis, and corneal ulceration—suggested a rubella infection. The finding of rubella antibody and an HAI titre of 512, and a CF titre of 32 six weeks after onset is good supporting evidence but virus isolation would have been better.

After the original febrile illness the subsequent course was clinically indistinguishable from active RA, as were the persistently positive tests for rheumatoid factor. It is therefore interesting that the patient recovered completely after 30 months, without clinical or radiographic residua. Systemic lupus erythematosus, rather than RA, seems unlikely in view of the persistently negative ANF tests. It is notable that during the period of the greatest clinical severity of the disease—with multiple joint pain and stiffness, synovial swelling and effusion—she had high antibody titres to rubella virus. It is also of interest that when she started to recover, and her joint symptoms and anaemia regressed and her erythrocyte sedimentation rate fell, the antibody titre to rubella virus declined *pari passu*.

Several authors (e.g., Haire and Hadden, 1970) have suggested that a persistent IgM antibody response to rubella virus, either in the congenitally infected infant or in young persons or adults, may indicate a continuing viral infection, perhaps with some concurrent viral immunosuppression. The results with our patient indicate a persisting rubella antibody response in the IgM fractions at least during the first $2\frac{1}{2}$ years after the (presumptive) attack of rubella. The persistence of complement-fixing antibody at 1/64 until December 1971 (i.e. 18 months after the onset of illness) also favours a persistent rubella infection. Failure to isolate or detect virus in the synovial fluid does not necessarily exclude its persistence in other cells or compartments of the joint cavity. London *et al.* (1970) have shown that in neonatally infected rabbits, rubella virus is found in highest concentrations in chondrocytes, but cartilage was not examined in this case.

The persistence of positive tests for rheumatoid factor for at least 18 months after the original illness also suggests continuing antigenic stimulation, perhaps by chronic rubella virus infection. Transiently positive tests for rheumatoid factor can occur after uncomplicated rubella infection but are generally infrequent. Kantor and Tanner (1962) found negative tests for rheumatoid factor in all 14 of their patients with rubella arthritis followed for up to 5 years. In their series of 5 patients with rubella arthritis, Chambers and Bywaters (1963) found a positive latex test in only one and the SSCT (DAT) was negative in all. However, Yanez *et al.* (1966) describe one patient in whom a tube latex titre of 1/5120 persisted for 16 months after the original rubella infection although the rubella arthritis had subsided within 2 weeks. Johnson and Hall (1958) reported positive rheumatoid factor tests in 9/10 patients with rubella arthritis but in only 2/7 patients with rubella uncomplicated by arthritis.

Broadly speaking, the findings in this patient could be interpreted in one of two ways. Either the rubella virus infection was the direct cause of the RA-like

illness, achieving its persistence by viral immunosuppression, or the abnormal response to the rubella virus infection uncovered or precipitated an inherent defect of T lymphocyte regulation of B cells, manifesting as an episode of rheumatoid arthritis with a prolonged IgM response to rubella virus but with eventual restoration of control. The evidence is inadequate to decide between these alternatives. Lymphocyte function as judged by phytohaemagglutination and pokeweed stimulation was normal after the patient had made a complete recovery but we do not know if this was so during the acute phase of illness. It may be significant however that there was no lymphocyte stimulation by candida antigen although the patient had had a candida infection of the vulva before and during the early stages of her illness, perhaps implying at least some defect in her cellular immune response. It will be important to follow the patient over several years to ascertain if there is a return of the RA-like illness without an antecedent virus infection.

Surveys of HLA antigens in congenital rubella (Honeyman *et al.*, 1975) suggest that the presence of A1, or a combination of A1 and 8, favours rubella virus infection. However, our patient lacked both A1 and A8 and genetic predisposition to persistent rubella infection cannot be inferred on these grounds.

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